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Regulation of Immune Functions in the Fetus and Newborn[1]

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Contents

I. Introduction

The timing of maturation of immunologic responses varies considerably in mammals with the capacity to respond to antigen appearing in utero for some species while in others immunocompetence is achieved only after birth

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[450]. Limited expressions f immune responsiveness can occur, for example, very early in fetal life as in the sheep, cow, m nkey, and man, or not until well int the postnatal period as is the case for the common laboratory rodents [430]. However, it is generally agreed that the developing fetus and newborn remain throughout the prenatal, and much of the neonatal period relatively immunoincompetent in comparison to the adult. The process of pre- and postnatal development from immunological immaturity to full immunocompetence appears to represent a well-ordered sequence of stepwise events leading to the gradual establishment of competence [430, 450]. Furthermore, a hierarchy of antigens has been shown to exist to which the developing young of a given species displays a characteristic capacity to respond at precise maturational stages [424, 430]. The position of an antigen in this hierarchy remains constant for the individuals within a particular inbred strain of mice, but varies between different strains suggesting a role for certain genetic factors in the acquisition of immune responsiveness during ontogeny [425]. Results from a number of different studies indicate that the orderly appearance of immunocompetence during ontogeny is not directly associated with the development of antigen-specific receptors on lymphocytes. Thus, there is good evidence that the repertoire of antigen-binding cells in adult and young animals is similar [119, 234, 431], and that these cells arise early in development at a time which may precede the ability of the animal to mount a specific immune response [108, 111].

It is now becoming increasingly clear that humoral and cell-mediated immune responses to most antigens are the result of a complex series of well-balanced macrophage-dependent T-T cell, and T-B cell interactions. Immunological homeostasis is maintained by complicated networks of feedback inhibitory cell circuits [69] in which macrophages would seem to serve to transmit T cell-derived communication signals to other T cells within a circuit [378]. The relative lack of responsiveness during ontogeny could thus be due to functional failures at one or more of the cellular interactions which occur in a normal immune response. An attempt will be made in the present article to relate what is presently known about immune deficiencies in the fetus and newborn to current concepts of induction and regulation of humoral and cell-mediated immunity.

While the immunological immaturity of the fetus and newborn does extend to both humoral [369] and cell-mediated reactions [202], ontogenetic studies frequently fail to reveal any obvious quantitative deficiency in the cellular constituents considered necessary to initiate an immune response [443]. These findings do not preclude, however, the likely necessity for further

qualitative changes in the lymphoid cell populations subsequent to the appearance of recognizable B and T cells with their apparent full range of antigen-binding specificities. It is notable in this regard that B lymphocytes undergo well-defined phenotypic shifts in their cell surface markers, including immunoglobulin, antigens coded by the Ia immune response genes and the complement C3 receptor [180, 181], as well as Lyb 3 [205], Lyb 5 [11] and MLs [12] determinants. Analogous time-dependent maturational events may have to occur in the antigen-presenting mechanism mediated by subpopulations of macrophages [105, 330, 331], and in functionally distinct subsets of T cells such as those defined by Ia [133] and by Lyt antigens [70, 133].

It is clear that both quantitative and qualitative changes in the lymphoid cell populations contribute to the emerging pattern of immune responsiveness during early ontogeny. However, there is now also a considerable amount of evidence that the developing young has an inherent capacity to respond to antigen much earlier than previously suspected, but that this potential for immune reactivity may be masked by dominating immunosuppressor mechanisms. One of the first indications for this come from a study [113] in which it was demonstrated that sensitized adult rabbit lymphoid cells give a relatively poor immune response when transferred t neonatal recipients wherease such cells were capable of strong antibody responses when transferred to irradiated adult recipients. Conversely, it was also shown that immune cells from neonatal rabbits did not function optimally in neonatal recipients, but they did when transferred to irradiated adult recipients [114]. The authors concluded that the immunological inadequacy of the neonate is probably related more to its inhospitable internal environment than to a lack of cells capable of antibody formation. More recently it has been shown that adoptive transfer of neonatal spleen cells to adult, lethally irradiated carrier-primed recipients maximizes the responsiveness of neonatal B cells [374]. This and other reports [279] indicate that while neonatal and adult cells demonstrate some quantitative differences in their responses, the relative immunodeficiency in newborn animals may not be primarily due to an inadequate population of responding B cells. Several other studies also suggest that the poor immune response of neonatal animals cannot be attributed solely to an underdeveloped antigen-processing system [138, 271, 379]. These findings together with the observation that newborn mice do not lack T helper cell function [379] lead one to conclude that neonatal incompetence is, at least in part, a manifestation of an overriding active humoral and/or cellular suppression.

It is well recognized that the maternal environment can exert consider-

able influence on the devel ping immune systems in the fetus and newborn [208, 263, 272, 438]. This article will therefore also be concerned with some of the immunoregulat ry aspects of the fetal/maternal relationship. The mammalian fetus can, according to the extensive studies of *Beer and Billingham* [38] be considered as an allograft at all stages of its existence. The total lack of maternal/fetal vascular communication as well as the presence of an effective neutral barrier (the syncytiotrophoblast) serve to distinguish the fetal allograft from conventional graft-host relationships [37]. However, the anatomical separation of maternal and fetal environment is incomplete and there is considerable bidirectional transplacental traffic of both humoral and cellular components [126]. The mutual sensitization which results from this exchange of antigenic material during gestation is thought to have both potentially harmful and beneficial effects on maintenance of the fetal allograft [39]. While it is now clear that several factors must be contributing to the success of the fetal allograft, there is much evidence to suggest that one important factor is a selective depression of the maternal immune system representing an adaptive response to protect the fetus from premature rejection [40].

The purpose of this article is (a) to describe the functional development of various cell types in the immune system during ontogeny, and (b) to examine the impact of naturally occurring maternal/fetal-newborn immunoregulatory forces on the emerging immune-response patterns. The literature cited has therefore been selected with this goal in mind and no attempt has been made to review comprehensively the immunobiology of the mammalian fetus and newborn. This review will concentrate on data obtained primarily in murine and to a lesser extent human systems since many of the relevant studies have been carried out in these species.

II. Ontogeny of T Cell Functions

A. Thymus

Thymus-derived T lymphocytes are involved, directly as effectors or indirectly as positive and negative regulators, in most types of specific immune reactions. T cells originate from fetal yolk sac, liver, or adult bone marrow-derived hematopoietic stem cells which migrate to the thymus via the bloodstream [351, 460]. Initial differentiation events within murine thymus involve the conversion of stem cells to thymocytes which are corticosteroid sensitive [49, 50], relatively immunoincompetent [50, 99], and bear

high density f Thy-1 [392] and TL [340] cell-surface antigens. Thy-1 and TL surface antigens have served as convenient markers to follow T cell development from immature cortical thymocytes to the more mature medullary T cells. The maturational process which occurs as thymocytes move from the cortex to the medulla [52, 411] results in the loss of TL antigens, a reduction in the amount of Thy-1 antigen, and the acquisition of immunocompetence. In general, medullary thymocytes resemble peripheral T cells with respect to Thy-1 density and relative resistance to irradiation and to corticosteroid treatment. Recent studies have shown that it is possible to compartmentalize the remarkably heterogeneous T cell populations into functionally distinct subsets on the basis of mitogen responsiveness [292, 455], rosette formation [412], Fc receptors [293, 457], the quantity of Thy-1 antigen [73], as well as cell surface Lyt [70, 198], Ia [57, 58, 211, 323, 466], and Qa-1 [445] antigen phenotypes. Many of these T lymphocyte subsets have stable cell-surface antigen phenotypes and are programmed for distinct functions [69]. It is clear that the ultimate manifestation of a T cell-dependent immune response is the result of a complex series of T-T synergistic [71, 72] and antagonistic [196, 395] interactions in which macrophages play an important intermediary role [378]. If one or more of the various effector and regulatory T cell subsets are out of normal physiological balance then an abnormal immune response is to be expected. Indeed, this has already been shown to be the case with the autoimmune disease-prone mouse strain NZB which has a defective Lyt 123^+ T cell subset [74], and in MRL mice which have a malfunction in the Lyt 1^+ subset [153].

The murine fetal thymus appears to contain thymocyte precursor cells as early as day 12–13 of gestation since these cells are capable of becoming lymphoid in organ culture [81]. The number of lymphoid cell precursors in the thymus at day 13 ranges from 7,000 to 15,000, and these cell numbers increase exponentially thereafter to reach a plateau by 18–19 days of gestation [216]. By day 19 of gestation most fetal thymocytes express approximately adult levels of Thy-1, Lyt 1^+, and Lyt 2^+ antigens [216]. While the peripheral T cells of neonatal mice are thought to be predominantly of the Lyt 12^+ phenotype [70] the cells which migrate out of the thymus of newborn mice are 60–70% Lyt 1^+ and 30% Lyt 12^+ [419]. A similar proportion of Lyt subsets has been reported for adult thymus migrants [419, 420] and for adult cortisone-resistant thymocytes [269, 419]. The neonatal thymocyte population has also been characterized as having a reduced number of small and medium size cells and an enhanced proportion of large, blast-like cells relative to the distribution of these cell types in the adult [325].

Mosier [300] and *Mosier and Cohen* [301] have studied the responsiveness of mouse thymocytes from day 16 of gestation through adulthood to various mit gens and alloantigens. Fetal thymocytes were found to be transiently more reactive in the MLR and to PHA than at any other time in the life of the animal. Reactivity to Con A and PWM increases gradually during the late fetal and early neonatal period and reaches adult levels between 2 and 3 weeks of age. Interestingly, the ability of neonatal thymocytes to respond to allogeneic cells in the MLR diminishes during the first 2 weeks of life but then returns to adult levels [300]. Additional studies by *Cohen and Mosier* [101] showed that hydrocortisone-resistant newborn thymocytes, which represents about 15% of the normal untreated newborn thymus, could account for all the PHA, Con A, and PWM reactive cells. In contrast, another recent report [397] finds that Con A responsiveness can first be demonstrated in murine fetal thymus at 18 days of gestation followed by the appearance of PWM reactivity at birth. In this study Con A reactivity rose slowly to reach a maximum after 2 weeks of age while PWM responses reached a peak within 1–2 days after birth. PHA responses remained low or negligible throughout the first 15 days of the post-natal period. The peak of PHA reactivity before birth as described elsewhere [323, 467] was not observed in this study. The weak or absent reactivity of newborn thymocytes to PHA [8, 71, 202] was reported to remain unchanged even after the administration of cortisone acetate to cell donors [202], a treatment which dramatically enhances adult thymic responses to PHA. In another comprehensive study of mitogen reactivity in the developing murine thymus *Byrd* et al. [63] also showed that newborn mice do not respond to PHA, but responded well to PWM. Reactivity to PHA was shown to occur later during young adulthood and reached a plateau by 8–12 weeks of postnatal life [202]. *Stobo and Paul* [456] showed that although the frequency of Thy-1-positive cells in newborn mouse thymus was equivalent to that of adult thymus, newborn thymocytes possessed very little reactivity to Con A. Thymocytes were shown to acquire Con A reactivity with age reaching a maximum at 4 weeks without either a change in the average number of cell-surface theta determinants, or any significant increase in the frequency of Thy-1-positive cells [456].

The appearance of MLR reactivity in rodent thymocytes is a very early pre- [323, 467] or postnatal [235] event, but the ability of T cells from the thymus to generate significant CML could not be detected until 1 week after birth [304]. However, while CML activity generated by neonatal thymocytes is weak, the lytic capacity per cell was found to be similar to that of adult lymphocytes. Studies on the ontogeny of graft versus host reactivity showed

that thymocytes developed the capacity to mount a GVH as measured in lethally irradiated adult recipients in the first few hours after birth [168]. The magnitude of the GVH reactivity by newborn thymocytes did not increase with age, as cells from 60-day-old animals had the same activity as similar numbers of cells from 3-day-old mice [168]. *Chakravarty* et al. [81] obtained similar results employing an in vitro GVH assay. They were unable to obtain GVH reactions with thymocytes taken at day 19 of gestation, but found that newborn thymocytes were as effective as adult cells in inducing splenomegaly in vitro [81]. *Chiscon and Golub* [88] studied the ontogeny of T helper cell functions in the thymus by determining the age at which thymocytes acquire the ability to collaborate with adult B cells in lethally irradiated recipients to produce an antibody response to SRBC. They found that this function remained less than 10% of adult values until birth. At birth T helper cell function increased exponentially, reaching adult levels within 48 h [88]. *Chakravarty* et al. [81] have also shown that day-16 embryonic thymocytes possess the capacity to collaborate with adult B in vitro to produce anti-SRBC antibody if the T cells were appropriately activated by 24 h of preincubation with Con A. In this system while the activity of day-16 embryonic thymus was low, the mitogen-activated thymic helper activity of day-18 embryos was identical to adult thymus [81].

The ontogeny of intrinsic immunocompetence in the thymus would, on the basis of the findings summarized above, seem to coincide approximately with the morphological appearance of lymphocytes. It is important to note, however, that these conclusions are based to a large extent on functional tests of developing lymphocytes in culture or in adult environments following adoptive transfer. The degree to which this reactivity is actually expressed within the physiology of the fetus and newborn will be discussed below.

B. Spleen

T lymphocytes found in characteristic thymus-dependent locations in pripheral lymphoid tissue are believed to largely originate from the small population of medullary thymocytes which account for most, if not all of the immunocompetence within the thymus [49, 50, 99, 101, 110, 254]. The ontogeny of thymus-derived T cell functions has been extensively studied in the murine spleen. *Spear* et al. [443] have determined the percentages of Thy-1-positive spleen cells as a function of age employing an immunofluorescence technique. T cells could be detected in the spleen as early as day 15 of gestation, and by day 16, 8% of the total nucleated cells were Thy-1-positive. Numbers of splenic T cells then increased rapidly until ap-

pr ximately 1 week after birth at which time they represented more than 75% of normal adult values. Other studies employing a cytotoxic assay based on chromium release [456] or immunofluorescence localization of T cells using rabbit antimouse thymocyte antibodies [145, 492] have reported considerably lower numbers of splenic T cells at birth. However, the high percentage of Thy-1+ cells found in blood, lymph nodes, and Peyer's patches at birth [82, 388] may support the claim that newborn mice possess a nearly adult frequency of T lymphocytes in the spleen [443]. There is very little increase in T cell numbers between 1 and 2 weeks of ages. Nevertheless, it is during this period that functional capacity of spleen cell populations begins to emerge in terms of mitogen [63, 202, 302, 442, 456] and alloantigen [9, 168, 202, 302] induced reactivity. Newborn spleen cell responsiveness to PWM was found to be substantial at 3 days of age, while significant reactivity to PHA was lacking until 17 days [202]. In this study both MLR and PWM reactivity was detectable in the spleen before PHA responsiveness. The refractoriness of neonatal spleen cells to PHA could not be overcome by varying the number of cells or the concentration of PHA. Kinetic studies by *Spear and Edelman* [442] showed that the number of spleen cells responding to Con A rose sharply from birth to 3 weeks of age, while PHA-reactive cells were not detected until 3 weeks of age and did not reach adult levels until 8 weeks. *Stobo and Paul* [456] reported that spleen cells from neonatal mice are virtually unreactive to Con A and PHA. They also demonstrated that spleen cells from 2-, 3- and 4-week-old animals came closer to the adult level of Con A responsiveness than of PHA responsiveness.

The response of spleen cells from mouse strains C57BL/6, CBA, and A/J to alloantigenic stimulation in MLR has been studied at closely spaced intervals from birth to 56 weeks of age [9]. No reactivity to alloantigens was present at birth. Following an unresponsive period lasting from 1 to 4 weeks depending on the strain combination, there was a sharp linear increase in alloreactivity which peaked at 14–20 weeks of age. However, in line with thymocyte data [235, 300, 301, 304] it was recently shown that spleen cells from newborn B10 mice are capable of responding to allogeneic stimulation by B10.D2 in primary MLC, whereas CML activity as measured by killing of PHA-stimulated B10.D2 target cells was not detectable until day 7 [498]. Poor CML by spleen cells from 4- to 5-day-old B10 mice could not be improved by varying the ratio of responding to stimulating cells indicating that the dichotomy of MLC and CML ontogeny was unlikely to be due to suboptimal culture conditions. Moreover, the ontogenetic sequence was not simply a reflection of a gradual increase in the magnitude of the MLC phase

of the CML. Thus, the MLC response was not greater in 13- to 16-day-old than 4- to 5-day-old animals, yet the lder mice demonstrated excellent CML while in the younger animals no CML was observed. *Pilarski* [367] subsequently developed a very efficient method for generating cytotoxic T cells which allowed the demonstration that the ability of spleen cells to generate CML in vitro had in fact begun to develop by 3 days after birth in Balb/c mice. By comparison, CBA spleen required 8–9 days of postnatal development before cytotoxicity levels reached the day-4 level of Balb/c mice. Addition of antigen-specific helper cells, considered to be required for [368] or at least helpful to [27, 100] the effective generation of CTL, did not augment CTL development in newborn mice. A lack of adherent/accessory cell activity in the newborn was shown not to be a limiting factor in these experiments [367]. The Lyt phenotype of alloreactive prekiller cells in the spleen of donors at increasing intervals after birth have been determined [61]. Spleen cells from 2-week-old B6 mice were shown to generate Lyt 23^+ cytolytic effector cells after stimulation with DBA/2 spleen cells. Lyt 123^+ cells are required for generation of Lyt 23^+ killer cells indicating that the phenotype of alloreactive prekiller cells at 1 week after birth could be Lyt 123^+. Examination of the Lyt phenotype of prekiller cells in progressively older mice revealed that at approximately 3–5 weeks of age the prekiller activity shifts from the Lyt 123^+ compartment to the Lyt 23^+ compartment [61].

CBA spleen cells from mice younger than 3 days of age were shown to be unable to elicit a significant GVH response in $B6AF_1$ mice [168]. In this study GVH reactivity was first evident at approximately day 4, and the response with a given number of transferred cells increased rapidly with increasing age of donor mice reaching adult levels within 14 days. In a separate study GVH reactivity was reported to be present in murine spleens after day 1 with full competence achieved as early as 4 days after birth [479].

Helper T cell function for T-dependent antibody synthesis does not reach adult-like activity until 4–5 weeks of age [443, 470]. Antibody responses to T-dependent antigens have been shown to occur earlier and with increased magnitude when helper T cell function is substituted or enhanced by mitogen [215, 329, 333, 414, 442] or thymus extract [53, 183]. The ontogenetic maturation of splenic T cells has also been followed by the ability to produce a T-cell replacing factor (TRF) which can restore T-dependent antibody responses in an in vitro T-cell deficient system [53]. Cultures of nude spleen cells could be stimulated to produce anti-SRBC antibodies in the presence of TRF obtained from adult cells but not from neonatal spleen cells. Only

after the mice were 1 to 2 weeks of age did their spleens have T lymphocytes which produced TRF [101].

Studies on the ontogeny of murine splenic T cells and their functions have been largely restricted to the neonatal period. This is a time when newborn animals will be presented with an array of foreign substances and infectious microorganisms. This may in part explain the sometimes quite different findings in the various functional assays in terms of the time of appearance of a particular immune reaction. We would conclude that very low numbers of T cells are present at birth in the spleen, but that they then rapidly increase to adult levels. Careful studies have also revealed that the neonatal murine spleen would seem to contain T cells of all known subsets but with different proportional distributions from adult spleen.

III. Ontogeny of B Cell Functions

B lymphocytes are derived from pluripotential hematopoietic stem cells [125, 497] which are themselves originally the progeny of primitive cells from the embryonic yolk sac [290]. Analysis of murine B cell development has indicated that the fetal liver is most likely the major site of cell differentiation in the embryo [365]. B lymphocytes normally develop multifocally in liver, spleen and bone marrow beginning about 3 days before birth [334, 352, 389]. Within a few days after birth the liver stops generating B cells [280, 403], and it is generally assumed that the differentiation of stem cells to B cells then shifts to the bone marrow [352]. Cells at the earliest recognizable stage in the B lymphocyte lineage are called pre-B cells. Such cells have cytoplasmic IgM, but no cell surface immunoglobulin detectable by immunofluorescence [244]. The actual time of appearance of murine pre-B and B cells varies somewhat from study to study depending on the particular definition of 'B cells', the method used to detect them, and the strains of mice tested. Employing immunofluorescent techniques *Raff* et al. [389] have described pre-B cells in murine fetal liver at 12 days gestation, approximately 5 days prior to the appearance of liver cells with detectable IgM on their surface [353]. *Melchers* et al. [281] have used sensitive biosynthetic labeling to demonstrate 7-8S IgM synthesis in fetal liver cells as early as day 11 of gestation, and the presence of surface-bound IgM on liver cells between days 12 and 15. B cells in the fetal liver [281, 403] and spleen [403] become mitogen reactive at birth, and by 7 days of age functional B cells in the spleen have reached 1/3 the adult level [403]. At about the same

time that fetal liver B cells become reactive to mitogen, they also become inhibitable by anti-immunoglobulin antibodies [281]. Newly developed IgM surface positive B cells in the fetus and newborn can be distinguished from their adult counterparts on the basis of sensitivity to anti-immunoglobulin-induced suppression [245, 264, 317]. Thus, *Raff* et al. [390] have shown that treatment with anti-IgM antibodies in vitro may induce irreversible modulation leading to permanent functional inactivation of immature B lymphocytes, while similar treatment of adult B cells has a reversible impact only. Antibodies synthesized by fetal and neonatal B cells have been shown in cell transfer studies using T-dependent antigens to be highly restricted with respect to their heterogeneity of avidity of the plaque-forming cells (PFC) compared with the PFC response of adult B cells [165]. Murine B cells acquire the capacity to produce an adult-like heterogeneous antibody response between 7 and 10 days of age. It was subsequently shown using another approach that as early as 14 days of gestation, B cell precursors in fact possess the genetic information required to synthesize an adult-like heterogeneous response [166]. The authors suggested that a differentiation event occurs between 7 and 10 days of age allowing the expression of a heterogeneous B cell response [166]; however, the contribution of an immunoregulatory process has not been ruled out. It has been shown by direct immunization of intact animals that young mice synthesize only IgM antibodies followed several days later by the capacity to synthesize significant amounts of IgG antibodies [80, 471]. However, cell transfer studies using syngeneic adult irradiated recipients supplied with an excess of adult T cells have shown that B cell precursors already have acquired the potential ability to produce IgG antibody by day 14–16 of fetal life [166].

The reason(s) for the poor antibody responsiveness of neonatal animals to conventional thymus-dependent antigens [138, 442, 443] cannot be attributed to any gross deficiency in numbers of Thy-1-bearing T cells and immunoglobulin-bearing B cells [443]. Thus, *Spear and Edelman* [442] and *Spear* et al. [443] noted that in spite of the prenatal appearance of sizable numbers of recognizable B cells and T cells in murine spleen, no detectable level of humoral antibodies occurs until after 1 week of age, and adult-like responsiveness is not achieved until 4–8 weeks of age. Moreover, several studies have clearly shown that the ability of newborn animals to respond to T cell-independent antigens precedes the capacity to produce antibodies to T cell-dependent antigens during ontogeny [186, 301, 305, 387]. The diminished capacity of the newborn to mount antibody responses may thus be partly due to a requirement for subtle postnatal maturational events result-

ing in changes in B cell functional properties. Indeed, it was earlier proposed [151, 370] and subsequently shown that several distinct B cell subsets exist which express individual response patterns to antigens which can be defined on the basis of cell-surface marker phenotypes [173, 214, 256, 257, 375]. It is possible to link these findings with ontogenetic studies which indicate that B lymphocytes proceed through several distinct maturational stages characterized by the expression of unique surface marker phenotypes at each stage [149, 182, 222, 417, 427, 485, 486]. For example, in the mouse from about 14 days of gestation until 7–3 days after birth, IgM is the only readily detectable isotypic marker on B cells [222, 417, 485, 487]. After 1 week of age a separate population of small splenic lymphocytes bearing both IgM and IgD [2, 170] appears and gradually increases until by 3 weeks of age 60% of spleen B cells exhibit this phenotype [222, 485]. As young adulthood is approached, at least three distinct populations of B cells exist in murine lymphoid tissues, namely cells bearing only IgM, those bearing both IgM and IgD, and cells bearing only IgD [486]. *Mosier* et al. [306, 307] have studied the functional consequences of the switch of B cell populations bearing primarily IgM to those bearing primarily IgD during ontogeny. It was noted that one group of T cell-independent (TI) antigens (i.e. the so-called TI type 1 antigens, which includes TNP-*Brucella abortus*) can effectively induce newborn spleen cells to produce specific antibodies in vitro. However, a second group of T-independent antigens (TI type 2, including TNP-Ficoll) was found to require a more advanced stage of B cell differentiation before antibodies could be induced. It was determined that the triggering process for responsiveness to TI 2 antigens requires interaction with an IgD $^{+}$B cell subset. The authors concluded from these findings that the acquisition of IgD is essential to the increase in immunocompetence expressed in developing young mice. In addition, other studies suggest that the phenotypic conversion of Ia^{-} B cells to Ia^{+} B cells is relevant to the changing response patterns during postnatal development [182]. *Lewis* et al. [257] have clearly shown that the expression of Ia antigens defines two subpopulations of adult B lymphocytes. The Ia^{-} primary B cell subpopulation contains precursors which give rise to only IgM antibody production, whereas the Ia^{+} subpopulation contains cells capable of also giving rise to IgG antibody synthesis. At birth IgM^{+} murine B lymphocytes are essentially all Ia^{-} [182, 222]. After birth, however, there is a linear increase in the percentage of IgM^{+} cells which express Ia, so that by 9 days of age 95% of splenic IgM^{+} cells are also Ia^{+} as is the case for adult spleen cells [222]. It is possible to speculate on the basis of these find-

ings that the capacity of the developing newborn to switch from solely IgM-producing capability to IgM plus IgG synthesis is to some extent linked to the appearance of an Ia+ B cell subpopulation. Finally, there is also some evidence that a C3 receptor surface marker may be important in determining the ontogenetic development of B cell activation [42]. The results of several studies have indicated that the C3 receptor is a component in the cellular pathway leading to T cell-dependent, but not T-independent antibody responses [137, 257, 362, 363]. Ontogenetic studies show that the C3 receptor-bearing subpopulation of IgM+ B cells does not appear in significant numbers until approximately 10 days of age. The appearance of a C3+ B cell subpopulation, therefore, would seem to correlate closely with the onset of capacity to produce antibodies to T cell-dependent antigens in the developing newborn.

There is little doubt that the development of immunocompetence in the newborn is to some considerable extent a reflection of qualitative changes in functionally distinct B cell subsets. However, as recently pointed out by *Mosier* et al. [307] the accumulated data on functional maturation of B cells suggest that by 2 weeks of age in the mouse, the state of B cell differentiation is probably not qualitatively limiting for antibody synthesis. The delayed appearance of adult-like immune reactivity to T cell-dependent and TI 2 antigens until 4–6 weeks of age would thus strongly suggest that additional factors are controlling the expression of immune responses during ontogeny.

IV. Ontogeny of Macrophages Involved in Immune Functions

Macrophages are known to play important roles in the induction and regulation of most types of humoral and cell-mediated immune functions [22, 287, 429, 481]. The selective depletion of macrophages from lymphocyte populations by plate adherence, carbonyl iron and magnet, antimacrophage serum plus complement, and by passage over Sephadex G-10 columns has been shown to drastically diminish or completely abrogate various immune functions [92, 247, 255, 303, 404]. Restoration of these immune reactivities could be accomplished by reconstitution with syngeneic macrophages. A more recently defined role for macrophages relates to the propensity of this class of cell to bind various T cell-derived regulatory factors [134, 136, 380, 469] and to transmit immunoregulatory signals to other cells [378]. Although both live and dead macrophages bind T cell factors, only live macrophages are able to successfully pass on the immunoregulatory

signals [154, 381]. On the basis of these findings the authors suggest that the role played by macrophages in relaying T cell communication signals is as important as their antigen-presenting functions. Macrophages are also known to produce their own immunosuppressive and stimulatory factors [64, 240]. Macrophage-lymphocyte interactions have been shown to be genetically restricted to the I region of the major histocompatibility complex [130, 405]. Moreover, macrophage populations which differ in their expression of Ia [106] may represent functionally distinct subsets involved in the induction of antibody synthesis [330] and in antigen-specific T cell proliferation [105].

Moore and Metcalf [291] have established that differentiated cells of the granulocytic series are absent in the murine yolk sac prior to 10 days gestation. In a study of the origin of cells of the mouse mononuclear phagocytic system, *Cline* [96] has shown that colony-forming cells (CFC) consisting of typical granulocytes and macrophages [97] can be generated in vitro in the 7- to 9-day-old yolk sac. Cell suspensions from embryos younger than 7–10 days did not form CFC. Fetal liver cells at 12–14 days of gestation were shown to give rise to functionally active macrophages following 6 days of culture in diffusion chambers [96]. Thus, mature macrophages as defined by phagocytic and immunoglobulin G receptor activity were observed in 10-day-old yolk sac cell suspensions and in 12-day-old fetal liver [96]. These findings suggest that the appearance of macrophages is a very early event in murine ontogeny. However, it has been suggested that the poor antibody responsiveness of newborn mice [138, 442, 443] is due in part to a lack of functional macrophages [21, 41, 48, 187]. This conclusion is based on findings that (a) neonatal mice are capable of increased antibody synthesis if given adult macrophages along with antigen [21, 41, 48], and (b) that peritoneal exudate cells (PEC) from newborn mice do not reconstitute antibody responsiveness to sublethally irradiated adult recipients while adult macrophages do [187]. However, with the possible exception of one particularly well-controlled study [48] attempts to attribute restorative effects of adult PEC's, known also to contain significant numbers of lymphocytes, solely to macrophage activity is questionable. Moreover, conclusions based on adoptive transfer of thioglycolate-induced neonatal PEC's [187] must be viewed in terms of earlier findings showing that adult PEC's induced with this agent failed to augment antibody responses in newborn mice [41], whereas unstimulated adult PEC were quite effective. *Landahl* [242] has shown that splenic adherent cells from newborn mice fail to synergize with adult splenic nonadherent cells to produce antibody until the 5th day after birth. The subsequent

increase in spleen adherent cell activity appeared to parallel the development of antibody responsiveness in the developing intact animal. These findings support the contention that a relative lack of macrophage function may be a contributing factor to the low levels of antibody synthesis in young animals. Other investigators, however, have concluded that the macrophages required for antibody synthesis are present in newborn mice [138, 387]. In reconstitution experiments in vitro employing reciprocal recombinations of adult and young macrophage-rich adherent cells and lymphocyte-rich nonadherent cells, it was found that the cellular deficit in young animals resided in the nonadherent lymphocyte-enriched fraction and not in the adherent population [138]. Also, *Rabinowitz* [387] demonstrated that addition of adult PEC to newborn spleen cell cultures failed to enhance either DNA synthesis or the primary plaque-forming cell response to SRBC. In addition, heat-killed macrophages, which themselves are incapable of antigen-processing, enhanced both the in vivo and in vitro antibody response of neonatal spleen cells [379] further indicating that the poor responsiveness of the newborn cannot be solely attributed to a defect in antigen processing and presentation. Using an antigen-specific macrophage-dependent T cell proliferation model *Lu* et al. [260] have recently presented evidence that the capacity of macrophages to interact with T lymphocytes effectively did not occur until 3–4 weeks after birth. The results suggest that the failure of macrophages from newborn mice to stimulate adult T cell proliferation is due to a relative deficit in numbers of a subpopulation of Ia-bearing macrophages [260].

Like most other cellular systems, macrophages have now also become subject to subset analysis. It is clear that only macrophages of a particular type with Ia-antigens expressed on their surface can function as proper antigen-presenting cells. Moreover, spleens of newborn mice are relatively deficient in the subpopulation of adherent Ia^+ dendritic cells [446, 447] which are known to be particularly effective stimulants of allogeneic [448] and syngeneic [336] mixed lymphocyte reactivity. Whether this is entirely because of a delayed development of such cells in young animals or due to the presence of other suppressing cells remains to be settled.

V. Immunoregulation in the Fetus and Newborn

A. Evidence for Suppressor Cell Activity

Ever since *Gershon and Kondo* [155, 156] first proposed that many immune responses are under the regulatory influences of thymus-derived T

lymphocytes, a prodigious amount of information has accumulated describing suppressor cell activities both in vivo [152, 288] and in vitro [120, 238]. Suppressor cell effects have been largely attributed to T cell populations [152, 155, 156, 288]; however, there is also evidence that B cells [117, 118, 221, 503] and macrophages [128, 129, 141, 142, 225, 232, 372] can mediate inhibitory functions. A variety of agents have been shown to activate suppressor cells including mitogens [116, 394], microbial substances [43, 59, 213, 413], anti-antibodies [127, 209], hormones [400], drugs [54], and chronic UV irradiation [140], in addition to alloantigens [89, 395] and a multitude of conventional laboratory antigens, when used at appropriate concentrations [68, 120, 121, 237, 238]. Many suppressor cell systems, once activated show exquisite specificity towards the stimulating antigen [152] while other spontaneous [32, 227, 489] or induced [62, 212, 326, 354] suppressor cells exhibit nonspecific effects. Murine suppressor T cells (Ts) have been shown in most experimental systems, to express the common cell surface antigen phenotype Lyt 1^-23^+, Ia^+ [278]. In some cases, however, inhibitory T cells have been reported to have an Lyt 1^+23^- [354, 391, 490] or Lyt 123^+ [366] phenotype. It is not yet certain whether these findings indicate the existence of truly nonconventional suppressor cell populations, with respect to Lyt phenotype, or that certain experimental designs inadvertently focused on an inductive or amplifying cell involved in the generation of conventional Lyt 1^-23^+ suppressors [69, 133]. In any case it is important to realize that the term 'suppressor cell' has taken on a more restricted and precise definition in view of recently obtained knowledge regarding the complexity of cell-cell interactions required to obtain the actual final effector(s) of suppression [69, 75, 122, 123, 273]. For example, immune Lyt 1 cells can help thymus-dependent B cells produce antibodies, and they can also serve as efficient inducers of Lyt 23 suppressor cells by interacting with a nonimmune set of Lyt 123^+ acceptor cells [75, 123, 273]. This regulatory pathway is known as a feedback inhibition circuit since one of the eventual targets for Lyt 123^+ cell-associated suppression is an Lyt 1^+ inducer cell [69]. Moreover, regulatory cells within or between such feedback circuits may communicate by secreting specific factors [218, 239, 296, 467, 504] which can be transmitted by macrophages [378] to the next cellular target in the sequence. Antigen-specific Ts [324, 338, 376] and Ts-derived suppressor factors (TsF) 174, 465, 472] have been shown to frequently bear determinants encoded by the I–J subregion of the major histocompatibility complex (MHC). It has been proposed that these I-region determinants serve as restrictive communicative links allowing the correct order of cell-cell interaction to take place within

an immunoregulatory circuit [468]. The chain of events in a regulatory loop extending from the original stimulus of a suppressor circuit to the ultimate feedback suppression of immune reactivity may involve the sequential activation of two or more sets of phenotypically distinct Ts and TsF [150, 468]. Thus, many of the 'suppressor cells' and 'suppressor factors' that have been previously described are probably not the actual effectors of suppression, but instead represent intermediaries of a long, complex chain of events leading to the final effectors of suppression. Recent analysis of Ts pathways in delayed-type hypersensitivity to the *p*-azobenzenearsonate (ABA) hapten has, for instance, revealed that the antigen-induced 'first order' suppressor cell bears a particular idiotype, while subsequent factor-induced 'second order' suppressor cells express anti-idiotype receptors [464]. This and other recent findings [124, 197] are in general agreement with the hypothesis that T cell circuits may establish idiotypic-anti-idiotypic regulatory pathways [464]. Neonatal mice are known to have a well-developed feedback-inhibitory circuit [318, 379] and lymphocytes from neonatal mice can suppress antibody responses of adult lymphocytes to phosphorylcholine via auto-anti-idiotype suppression [458]. It is therefore likely that fetal/neonatal immunoregulatory pathways may prove to be as precise and complex as those presently being defined in the adult.

1. Suppression of Antibody Synthesis

As previously noted newborn mice acquire the ability to produce antibodies to most T cell-independent antigens at a very early stage in postnatal development [112, 186, 277, 301, 305, 307, 387], while responsiveness to T cell-dependent antigens usually appears later [138, 186, 387, 442, 443, 470]. *Mosier and Johnson* [305] initially interpreted these findings as indicating that B cells mature earlier during ontogeny than do T cells in terms of their functional roles in antibody formation. Their experiments to test this reasoning in which an excess of adult lymph node cells were added to antigen-stimulated neonatal spleen cell cultures showed, however, that mature adult T cells could not restore the poor responsiveness of newborn spleen. Prior removal of T cells from neonatal spleen with anti-Thy-1 and complement was necessary to allow the remaining neonatal B cells and macrophages to respond in culture when excess adult T cells were added. In addition, small numbers of neonatal spleen cells were shown to significantly suppress antibody responses of adult spleen cells to the T cell-dependent antigen, SRBC as well as to the T-independent antigen DNP-Ficoll. The suppressive effect by newborn spleen cells was found to be associated with cells passing

through nylon wool columns, and was abrogated by anti-Thy-1 and complement treatment. From these findings it was concluded that the poor antibody responsiveness in newborn animals is at least partly due to an excess of endogenous suppressor T cells which can interfere with helper T cell functions and also negatively influence T-independent B cell functions [305]. This conclusion was strengthened by subsequent studies showing that neonatal thymocytes constitute a rich source of suppressor cells for adult antibody synthesis [308]. A considerable enrichment of mouse thymus-derived suppressor cells was achieved by infection of newborn animals with mouse thymic virus [102]. The residual T cells remaining after infection, representing about 5% of the normal thymus T cell number were highly enriched for suppressor activity and retained PHA and ConA reactivity, but had lost all ability to respond in MLC and CML assays [308]. The population of mouse thymic virus-resistant T lymphocytes containing the neonatal inhibitory cells were characterized as large cells with high DNA content and were localized to the subcapsular outer cortex of the thymus. In addition, these cells were found to be less susceptable to killing with anti-TL and anti-Thy-1 sera and more susceptable to killing with anti-H-2^b serum that was the average normal thymocyte. It was inferred in these studies that suppressor cells residing within the residual 5% virus-resistant outer cortical thymocytes emigrate to the neonatal spleen where similar immunosuppressive activity was detectable [308].

The thymic origin of the nonspecific suppressor cells which appear early in ontogeny was further substantiated by studies showing that unprimed fetal thymocytes efficiently suppressed the T-dependent primary antibody response of adult syngeneic spleen cells to SRBC [261]. However, contrary to the mouse thymic virus-resistant suppressors which appeared to be of cortical origin [308], the fetal thymus-derived suppressor cells demonstrated characteristics of a thymic medullary lymphocyte.

The fetal suppressor T cell was shown to have a low content of Thy-1 antigen and was not sensitive to treatment with hydrocortisone [261]. Studies by *McCullagh* [270, 271] performed in the rat also indicate that antibody responsiveness during the neonatal period is regulated by an active thymus-derived T cell suppressor mechanism. It was demonstrated that the impaired capacity of newborn rats to mount an anti-SRBC response could be restored by adoptive transfer of adult thoracic duct lymphocytes only if the neonatal recipients were first thymectomized [270] or exposed to low dose of irradiation [271]. Antibody-forming cell precursors were shown to be plentiful in the newborn rat. These findings were interpreted as indicating

that the failure of newborn rats to sustain adoptive responses was not due to inadequate neonatal macrophage function, but to an active newborn regulatory mechanism mediated by radiosensitive suppressor T cells of thymic origin [270, 271]. The suppressor T cells residing in murine neonatal spleen and thymus have also been characterized by *Mulder* et al. [310] as being sensitive to both X-irradiation and mitomycin C. *Calkins and Stutman* [65] have presented evidence suggesting that the suppressor cell population in newborn mice which inhibits antibody synthesis of adult lymphocytes in culture [301, 305] is distinct from the suppressor cells which can be found in adult lymphoid tissues [66, 122]. Newborn suppressors were thus shown to differ from adult inhibitory T cells in terms of an apparent absence of antigen specificity, lack of requirement for stimulation from primed cells to suppress, and a relative insensitivity to cytotoxic treatment with anti-Thy-1 and complement.

There is at present very little detailed information concerning the mechanism(s) of action of the fetal and neonatal cells which inhibit antibody synthesis. There is some evidence to suggest that neonatal suppressors act in vitro during the early inductive phase of the antibody response [261] and that suppression may be by cell contact rather than through the elaboration of soluble suppressive factors [310]. The question of whether the primary target for neonatal T suppressors is a B cell or another T cell has not been resolved. *Mosier* et al. [308] presented evidence that neonatal T cells can exert a negative regulatory influence directly on B cells responding to the T cell-independent antigen TNP-Ficoll by reducing the number of B cells initially triggered by antigen. Neonatal T cells have also been shown to suppress other T-independent antigens including, type III pneumococcal polysaccharide [294], the synthetic polypeptide poly-(*D*-Tyr, *D*-Glu)-poly (*d*-Pro)-poly (*D*-Lys) [188], and DNP-PAA (polyacrylamide beads) [112]. However, studies involving careful dilution analysis of suppressor T cell effects on T-dependent versus T-independent antigens indicate that T-dependent antibody synthesis is much more sensitive to suppression by fetal or neonatal cells than is T-independent antibody synthesis [261, 318]. Thus, *Luckenbach* et al. [261] have shown that fetal thymocytes suppress the in vitro antibody response of adult spleen against SRBC when present in thymus to spleen cell ratios of 1:5 to 1:500, whereas no suppression was observed on the antibody response to the TI antigen NIP-POL even at a fetal thymocyte to adult spleen cell ratio of 1:5.

We have examined the immunoregulatory effect of purified splenic T lymphocytes from newborn mice on in vitro antibody synthesis to the thymus-dependent antigens SRBC and DNP-KLH, and on the thymus-

independent antigens DNP-Ficoll and DNP-KLH [318]. In these experiments T lymphocytes were isolated from the spleens of 5- to 8-day-old or 6- to 8-week-old adult CBA/H mice by affinity fractionation on Ig-anti-Ig glass bead columns [493]. The purified T cells were then added in graded numbers to assay Marbrook cultures containing 20×10^6 normal adult CBA/H spleen cells and optimal immunizing doses of TD and TI antigens. Antibody synthesis was measured after 4 days of culture as antigen-specific IgM PFC. The results presented in table I show that the addition of 1×10^6 newborn spleen T cells, representing 5% of the total cell population in the assay culture, strongly inhibits antibody synthesis to the two TD antigens, SRBC and DNK-KLH. Significant inhibition of TI antibody synthesis was observed in only a few instances (2 out of 7 experiments) and then only when the ratio of newborn T to adult spleen cells was 1:20 or higher. Thus, dilution analyses showed that suppression of TI responses occurred only in the presence of relatively high numbers of newborn T cells, whereas TD responses were consistently inhibited by 10^4 (0.05% of the total cell population in assay cultures) and sometimes by as few as 10^3 newborn T cells. It is notable that the suppression of TI responses by newborn lymphocytes in vitro reported by others [112, 308] was shown only to occur at a ratio of newborn to adult cells much higher than 1:5. It is our experience that normal adult T cells begin to exert inhibiting effects in control cultures when added in excess of 20% of the total cell population in the present culture system. Nevertheless, we conclude that it is likely that newborn T cells, and/or an as yet undefined contaminating cell subpopulation, can exert inhibitory effects on TI antibody responses but with low efficiency. However, the prevailing inhibitory effect mediated by newborn T lymphocytes on antibody synthesis in vitro is directed towards TD responses. These findings could be interpreted as indicating that newborn inhibitory T cells either directly suppress TD B cells more efficiently than they do TI B cells [257], or that helper T cells required for TD antibody synthesis are primary targets for suppression. Our preliminary evidence tends to favor the latter possibilty. Thus, newborn T cells have been shown to exert helper effects for TD antibody synthesis when added to adult T cell-depleted spleen B cell cultures, while causing strong suppression of antibody production in parallel cultures of whole adult spleen cells [*Stegagno and Murgita*, unpublished observations].

Negative selection experiments were performed with various anti-T cell reagents plus complement to determine the cell surface antigen phenotype of the newborn inhibitory T cell. Purified splenic T cells from 5-day-old

Table I. Inhibitory effect of newborn splenic T lymphocytes on T cell-dependent versus T cell-independent antibody responses in vitro

Source of splenic T cells Added to assay culture	Assay culture: number of T cells transferred to assay culture	I_gM PFC/culture ± SEM (% suppression)			
		SRBC-PFC	DNP-specific PFC		
			DNP-KLH	DNP-Ficoll	DNP-POL
–	–	1,140 ± 260	1,181 ± 74	768 ± 79	1,176 ± 63
T_{adult}	10^6	1,146 ± 96 (0)	1,758 ± 38(0)	952 ± 79(0)	1,740 ± 82 (0)
$T_{newborn}$	10^6	452 ± 35 (60)	591 ± 45(50)	460 ± 36(40)	1,140 ± 95 (3)
$T_{newborn}$	10^5	653 ± 148(43)	678 ± 34(43)	695 ± 20(10)	1,127 ± 54 (4)
$T_{newborn}$	10^4	690 ± 80 (39)	520 ± 26(56)	817 ± 44(0)	1,165 ± 66 (0)
$T_{newborn}$	10^3	1,180 ± 160(0)	673 ± 22(42)	830 ± 40(0)	1,060 ± 772(8)

Ig-anti-Ig column purified splenic T cells [493] from 5- to 8-day-old CBA/H mice were added in graded numbers to secondary Marbrook assay cultures containing 20×10^6 normal adult CBA/H spleen cells and optimal immunizing doses of the T cell-dependent antigens SRBC (3×10^6), and DNP-KLH (0.1 μg/ml), and the T-dependent antigens DNP-Ficoll (10 ng/ml) and DNP-POL (1 μg/ml). Antigen-specific IgM plaque-forming-cell (PFC) responses were measured after 4 days of culture. Data are expressed as the mean number of PFC ± standard error of the mean of triplicate cultures. DNP-specific PFC were enumerated using TNP coupled SRBC as indicator cells [see reference 314 for details].

Table II. Cell surface Lyt differentiation antigen phenotype of newborn splenic inhibitory T cells

Source of splenic T cells	Cytotoxic treatment with antisera and complement	Assay culture, % of control PFC response (mean ± SEM of 5 experiments)
–	–	
T_{adult}	–	99 ± 5
$T_{newborn}$	–	44 ± 2
$T_{newborn}$	+ RC	46 ± 2
$T_{newborn}$	anti-T + RC	98 ± 5
$T_{newborn}$	anti-Thy-1.2 + RC	75 ± 2
$T_{newborn}$	anti-Ly 1 + RC	99 ± 7
$T_{newborn}$	anti-Ly 2 + RC	42 ± 3

Ig-anti-Ig column purified splenic T cells from newborn CBA/H mice were exposed to anti-T (rabbit anti-mouse brain antiserum), anti-Thy-1.2 (AKR anti-C3H), anti-Lyt 1.1 and anti-Lyt 2.1 antisera plus rabbit complement (RC). Following selective lysis with these antisera the remaining cells were adjusted such that the lymphocyte density in the untreated control was 1×10^5 viable cells per ml. One ml aliquotes of cells were transferred to adult CBA/H spleen cell assay cultures immunized with SRBC. The number of day-4 IgM anti-SRBC PFC in control cultures without added T cells was 665 ± 56.

CBA/H mice were exposed to rabbit antimouse brain (anti-T), anti-Thy-1, anti-Lyt 1.1 and anti-Lyt 2.1 sera plus rabbit complement. Following selective lysis with these antisera the remaining cells were tested for their ability to induce suppression of TD anti-SRBC response in secondary assay cultures. As shown in table II, the ability to cause suppression was eliminated by cytotoxic pretreatment with anti-T, anti-Thy-1, anti-Lyt 1.1, but not by anti-Lyt 2.1 sera. Absorption studies verified that the elimination of suppressor activity was, in fact, due to the effect of specific anti-Lyt 1.1 antibodies, and additional phenotyping experiments were performed with other mouse strains with essentially identical results [318]. Thus, lymphocytes capable of inhibiting TD antibody responses residing in the spleen of newborn mice are T cells with an Lyt 1^+2^- phenotype [167, 318]. We further analyzed the Ia antigen phenotype of these newborn inhibitory T cells, as such markers have been found to be of importance in delineating various T cell subsets in other systems [133, 323, 339, 466]. Splenic T lymphocytes from 2- to 4-day-old CBA/J mice were exposed to anti-T cell sera, anti-$H\text{-}2^k$, and to various anti-Ia sera specific for subregions within MHC I re-

and DNP-KLH (0.1 μg/ml) and T-dependent antigens DNP-Ficoll (10 ng/ml) and DNP-POL (1 μg/ml). Antigen-specific IgM plaque-forming-cell (PFC) responses were measured after 4 days of culture. Data are expressed as the mean number of PFC ± standard error of the mean of triplicate cultures. DNP-specific PFC were enumerated using TNP coupled SRBC as indicator cells [see reference 314 for details].

gion. After selective lysis in the presence of rabbit complement the cells remaining were tested for their ability to suppress anti-SRBC antibody synthesis of adult normal spleen cell cultures. The results presented in table III show that the inhibitory activity of newborn T cells is effectively abrogated by anti-I^k, anti-I-ABJ^k, anti-I-J^k, but not by anti-I-EC^k, or by anti-I-J^b. The I-J^k determinant is therefore expressed on newborn CBA/J splenic inhibitory T cells [200, 319].

While the presence of an I-J marker is known to be associated with T cells possessing suppressor properties, the expression of the Lyt 1^+2^- phenotype on the newborn inhibitory T cells is not compatible with conventional suppressor effector cells which are usually Lyt 1^-2^+ in phenotype. It is likely, although as yet unproven, that the Lyt 1^+2^- newborn inhibitory T cells represent a population of 'suppressor inducer' cells, rather than being truly non-conventional effector suppressor cells. This premise is based on evidence by *Ptak* et al. [379], that newborn mice can be shown to contain considerable numbers of 'latent' helper cells when overriding suppressor mechanisms are deliberately interrupted. Moreover, this study revealed distinct similarities between neonatal helper cells and a subpopulation of adult helper T cells which have powerful suppressor-inducing abilities. These findings would therefore indicate that neonatal Lyt 1^+2^-, I-J^+ regulatory T cells may be comparable to a certain type of adult Lyt 1 helper cell which is an efficient inducer of feedback inhibition [69]. An alternative possibility, albeit less likely, is that the newborn Lyt 1^+ inhibitory T cell we have described is an actual specialized effector cell which operates outside previously described feedback circuits [69] to help control immune reactivity during ontogeny. It is interesting to note in this regard that while young NZB mice show an unusually early appearance of immunocompetence [132, 369], which may be linked to an absent Lyt 123^+ cell-associated feedback suppression circuit [76], newborn NZB spleen cells still retain active inhibitory cell activity as measured in vitro [246].

There is now substantial evidence for the presence in newborn mice of an unusually active population of T cells with efficient ability to suppress T cell-dependent antibody synthesis in vitro. While the mechanism of suppression may be direct or indirect, it is likely that helper T cell function is a primary target for the inhibitory process. Whether these inhibitory cells differ in number or activity per cell from similar T cells in adult individuals is not known. Newborn T cells would also seem able to exert less effective suppressive activity on certain relatively-T-independent antibody responses. The actual reason(s) for the presence of active inhibitory cells in the neonate

Table III. Expression of I-J determinants on CBA/J newborn splenic inhibitory T cells

Source of T cells	Treatment antiserum + RC	H-2 region detected								Assay culture I_gM SRBC PFC/ culture ± SEM (% suppression)
		K	I-A	I-B	I-J	I-E	I-C	S	G	D	
—	–										652 ± 47
T_{adult}	–										886 ± 62(0)
$T_{newborn}$	–										384 ± 55(41)
$T_{newborn}$	RC										360 ± 50(45)
$T_{newborn}$	anti-T										748 ± 64(0)
$T_{newborn}$	anti-Thy-1										576 ± 76(12)
$T_{newborn}$	C3H. SW anti-C3H	K	K	K	K	K	K	K	K	K	732 ± 12(0)
$T_{newborn}$	A. TH anti-A. TL		K	K	K	K	K	K	K		840 ± 81(0)
$T_{newborn}$	(A. THXBIO. HTT) F_1 anti-A. TL		K	K	K						780 ± 40(0)
$T_{newborn}$	BIO. A (3R) anti-BIO. A (5R)				K						756 ± 43(0)
$T_{newborn}$	BIO. S (7R) anti-BIO. HTT					K	K	K	K		372 ± 21(43)
$T_{newborn}$	BIO. A (5R) anti-BIO. A (3R)				B						384 ± 52(41)

Ig-anti-Ig column purified splenic T cells from 2- to 4-day-old CBA/J mice were treated with anti-Ia subregion specific alloantisera (prepared at the University of Toronto by Dr. T. L. Delovitch) plus rabbit complement (RC). The cells remaining after cytotoxic pretreatment were adjusted to a viable lymphocyte count of 10^6 per ml in the untreated control. Aliquotes of pretreated T cells, containing the equivalent of 10^6 cells minus the number selectively killed, were transferred to assay cultures consisting of 20×10^6 syngeneic adult spleen cells plus 3×10^6 SRBC. Day-4 IgM anti-SRBC PFC are expressed as the mean number of PFC ± standard error of the mean of triplicate cultures.

is still a matter of speculation. In addition, we feel that the presence of other non-T suppressor cells has not been fully excluded by the available published evidence.

2. Suppression of Cell-Mediated Immunity

Olding and Oldstone [341] and *Olding* et al. [343] first observed that lymphocytes from cord blood of human newborns could effectively inhibit division of their mother's lymphocytes in vitro. In these studies equal numbers of viable lymphocytes from a mother and her male baby were mixed and cultured for 3 days with and without PHA. The mitogen was usually needed to get a sufficiently high number of dividing cells for chromosome analysis. Examination of mother-baby mixed cultures for the occurrence of male and female metaphases using the fluorescent Y chromosme technique revealed that 98% of the total cultured lymphocytes in mitosis were the newborn's, whereas less than 2% were from the mother [341, 343]. The same strong mitotic inhibition also occurred when lymphocytes from newborn male babies were mixed with lymphocytes from nonrelated recently delivered mothers and from nonrelated, nonpregnant women. *Lawler* et al. [243] reported a similar inhibitory effect by male newborn lymphocytes on parental cells in two-way mixed cultures. Subsequent cell fractionation studies showed that the suppressor activity in cord blood was associated with the T lymphocyte population [342].

We have further examined the immunologic consequences of the interaction between lymphocytes from human newborns and their mothers in vitro [344]. The cellular proliferation of mother-baby in two-way mixed lymphocyte reactions was assessed by simultaneously measuring ^{3}H-thymidine incorporation and the number of cells in mitosis containing the Y chromosome. The results demonstrated that the mitotic inhibition of maternal cells can be readily detected in 6-day two-way mixed cultures of equal numbers of newborn and maternal lymphocytes. Approximately 94–96% of the total cultured cells contained the Y chromosome, indicating that most of the dividing cells in the cocultures were of newborn origin. As shown in table IV, the strong suppressive effect mediated by actively dividing newborn lymphocytes was converted to a stimulatory effect on maternal lymphocyte proliferation by irradiation of the newborn cells. We also sought to determine whether the mode of action of newborn inhibitory lymphocyte was via cell-cell contact or through the liberation of a soluble inhibitor factor(s). Experiments were performed using double chamber Marbrook cultures [135] in which newborn suppressors and adult target lymphocyte

Table IV. Lymphocytes from human newborns must be viable to suppress proliferation of their mother's lymphocytes in the mixed lymphocyte reaction

Case No.	Culture time, days	Uptake of ^{3}H-thymidine by lymphocytes from			% of metaphase cells having a Y chromosome	Stimulation index
		mother	newborn male	mother + newborn		
1	6	2,734 ± 209	7,935 ± 501	18,988 ± 1,534	96	1.8
2	6	1,050 ± 178	2,323 ± 613	10,662 ± 985	94	3.2
3	6	3,554 ± 328	*447 ± 12	21,699 ± 1,724	0	5.4
4	6	1,893 ± 144	*380 ± 129	14,919 ± 1,456	0	6.6

* Lymphocytes from newborn male babies were irradiated with 6,000 R before co-culture with maternal cells.

Lymphocytes, obtained from cord blood of newborn male babies and from peripheral blood of their mothers, were enriched by Ficoll-Isopaque gradient centrifugation, and suspended in RPMI medium supplemented with 10% pooled AB serum. 2×10^5 lymphocytes from the mother and baby were cultured alone, and co-cultures contained 1×10^5 cells from each individual. For Y chromosome analysis cells from multiple cultures were pooled, treated with colchicine for 2 h, disrupted, fixed, and stained with quinacrine-dihydrochloride [for details see *Olding* et al. 343]. Maternal lymphocytes survived as well as newborn lymphocytes in this culture system, and maternal cells cultured alone were able to enter mitosis normally. Cases 1 and 2 demonstrate a mitotic inhibition of maternal cells as the result of co-culture with an equivalent number of viable baby lymphocytes. In cases 3 and 4 the inhibitory effect of newborn lymphocytes on maternal cell proliferation is abrogated when baby cells are irradiated with 6000 R prior to co-culture.

could be separated by Nucleopore or dialysis membranes. PHA was added to both lymphocyte compartments. After 3 days of culture, the newborn cells in the inner chamber, and maternal cells in the outer chamber of Marbrook vessels were pulsed with ^{3}H-thymidine. The amount of radioactivity incorporated by maternal lymphocytes exposed to the dialyzable products of newborn lymphocytes was compared to control cultures consisting of maternal cells cultured alone. Our findings summarized in table V show that mitogen-stimulated cells from newborns could suppress the proliferation of lymphocytes from recently delivered mothers even when separated by a dialysis membrane. PHA-stimulated lymphocytes from nonrelated adults did not inhibit division of maternal lymphocytes, or lymphocytes from another nonpregnant woman when physically separated in double chamber cultures. Moreover, lymphocytes from one newborn failed to inhibit the proliferation of lymphocytes from another newborn. Our findings thus indicated that proliferating newborn lymphocytes induce mitotic inhibition

Table V. Human newborn lymphocytes secrete a low molecular weight factor inhibitory for maternal lymphocyte proliferation

Case No.	Lymphocytes from	Exposed to dialyzable product(s) from	^{3}H-thymidine incorporation (cmp ± SEM)	% suppression
1	mother	–	148,697 ± 19,123	
		newborn	42,135 ± 514	71
2	mother	–	106,454 ± 1,658	
		newborn	54,987 ± 873	48
3	mother	–	107,225 ± 4,298	
		newborn	43,829 ± 1,254	59
4	mother	–	81,945 ± 2,156	
		nonpregnant female	75,515 ± 988	8
5	nonpregnant female	–	191,180 ± 1,957	
		nonpregnant female	231,615 ± 1,898	0
6	newborn	–	265,179 ± 9,426	
		newborn	267,792 ± 4,970	0

Using a Marbrook double chamber culture system [344] 4×10^6 newborn lymphocytes were separated from 8×10^6 maternal lymphocytes by a dialysis membrane. PHA was added to both compartments in a concentration of 50 μg/ml. After 3 days of culture th cells in both compartments were harvested and 1×10^5 viable lymphocytes were transferred to microplate wells and pulsed for 5 h with ^{3}H-TdR. In all experiments the survival rates of the cells in both chambers were equivalent.

of adult cells by releasing a low molecular weight suppressive factor(s). Other studies have also subsequently shown that cord blood lymphoid cells secrete soluble factors capable of inhibiting mitogen and alloantigen-induced proliferation of adult cells [495, 496]. *Oldstone* et al. [345] have further characterized the inhibitory cells in cord blood as a T lymphocyte subset bearing Fc receptors for IgG(T_G). This is consistent with findings that adult T lymphocytes with receptors for IgM(T_M) act as helper cells, while those with IgG receptors are efficient suppressors [293], and that the mean proportion of T_G cells in cord blood is about 3 times the adult mean [345]. More recent studies [115] indicate that the suppressor pathways in the human newborn may be more complex than earlier believed. Thus, newborn T_G^+ cells suppress B cell maturation, but not B cell proliferation, while newborn T_G^- cells are strongly suppressive for T and B lymphocyte proliferation. Moreover, PWM-induced newborn suppressor T lymphocytes only inhibit the maturation of adult B cells when other T cells were present,

suggesting that the newborn 'suppressor' is capable of inducing a secondary suppressor activity in the adult T cell population. It is possible that one of the inhibitory T cell subsets present in cord blood [115] may be analogous to the Lyt 1^+ suppressor inducer cell in the murine feedback inhibition circuit [69].

The embryonic liver is known to be an early site of immunocompetence during murine ontogeny. *Globerson* et al. [163] have examined the question of whether the potential to suppress immune reactivity arises concomitantly with the appearance of immunocompetence in the liver. In initial studies mouse embryonic and neonatal liver cells were found to strongly suppress adult mixed lymphocyte reactivity and generation of cell-mediated lysis. The suppression occurred regardless of whether stimulator cells were syngeneic or allogeneic to the liver donor [163]. Newborn liver cells also suppressed GVH-induced mortality in sublethally irradiated F_1 mice challenged with parental spleen cells mixed with parental liver cells [162, 480]. Interference with GVH responses by neonatal liver cells could be demonstrated only in combinations where the injected spleen cells were syngeneic to the inhibitory liver cells. This genetic restriction for suppression of GVH in vivo contrasted with the findings earlier where MLR was inhibited by liver cells syngeneic or allogeneic with the effector cells [163]. Mouse embryonic liver suppressor cells have been characterized as belonging to a subpopulation of cells bearing receptors for the lectin peanut agglutinin [386].

Fetal mouse splenic, but not thymic cells have also been shown to reduce the ability of parental adult spleen cells to cause local graft-versus-host reactions in F_1 mice [432]. Suppression was still observed with spleen cells from newborn mice less than 24 h old, but disappeared completely by the time mice were 5 days of age. There was no requirement for histocompatibility between reacting and suppressor cells. It was inferred that the splenic suppressors were T lymphocytes since the inhibitory activity was completely eliminated by pretreatment with anti-Thy-1 serum [432]. In further studies by *Ptak and Skowron-Cendrzak* [377] it was shown that newborn mice sensitized to picryl chloride (PCL) within 24 h of birth fail to develop contact sensitivity when tested 4 weeks later. The reaction of animals sensitized at 2 days of age or later was always greater than in non-sensitized controls. It was postulated that painting with PCL at birth generates specific suppressor cells which after 4 weeks can still specifically interfere with the ability of the animals to respond to the original skin sensitizer [377]. Spleen lymphocytes of fetal or 1-day-old newborn mice were shown to suppress the passive transfer of contact sensitivity when injected along

with sensitized lymphocytes. Thymocytes were unable to affect the transfer reaction regardless of age of the cell donor, or the cell number used. Fetal mice were shown not to develop GVH reactions when given injections of parental lymphocytes. Moreover, fetal spleen cells were able to suppress the local GVH reaction elicited by immunized parental cells in F_1 recipients, and to reduce the severity and mortality rate of GVH in cyclophosphamide-treated F_1 recipients. These findings taken together imply that while antigen given in the perinatal period does not cause a specific immunization or sensitization, it may result in the triggering of an efficient antigen-specific suppressor mechanism.

Bassett et al. [34] have studied the inhibitory properties of newborn mouse splenic lymphocytes on a semiallogeneic mixed lymphocyte reaction. It was observed that stimulation of adult parental responder lymphocytes by newborn F_1 splenic lymphocytes was much lower than by genetically identical adult F_1 cells. The lack of stimulation was found not to be due to inadequate expression of MLR-activating antigens on the surface of newborn cells. Thus, mixtures of newborn and adult F_1 stimulator cells resulted in a reduction of proliferation of adult parental cells comparable to that observed when using newborn stimulators alone. It was concluded that newborn mouse spleen contains a suppressor which can inhibit adult responder cells in the MLR. Additional experiments showed that newborn F_1 cells cultures alone produce a factor capable of suppressing an adult parental anti-F_1 MLR [34]. These results were subsequently confirmed and extended by *Pavia and Stites* [356]. Their study also showed that irradiated newborn F_1 spleen cells failed to inhibit the proliferative response of maternal strain mice to PHA and Con A. Responses to LPS were suppressed by 55–60% compared to control cultures containing LPS-stimulated maternal cells alone. The newborn spleen suppressor activity for the semiallogeneic MLR was shown to persist for the first 2 ½ weeks after birth and thereafter decayed quickly. The inhibitory activity of newborn spleen cells for the MLR was sensitive to cytotoxic pretreatment with heterologous antibrain associated T antigen [169] plus complement indicating that the suppressor is a T cell. Newborn spleen cells which did effectively suppress MLR were not able to interfere with the in vitro generation of cytotoxic lymphocytes in the CML assay. It was therefore suggested that newborn suppressor cells can selectively inhibit certain lymphocyte subpopulations involved in proliferative, but not cytotoxic reactions.

Argyris [23] has shown that T helper cells present in adult but not newborn spleen are effective in allowing in vitro proliferation of low numbers of

adult mouse thymus cells to allogeneic spleen cells in vitro. Helper activity was totally absent in 6- to 8-day-old newborn spleen, partially present in 20-day-old spleen cells, and reached adult levels in the spleen of 40-day-old mice. Mixing neonatal spleen cells with adult spleen helper cells eliminated the adult helper activity for thymus-responding cells in the MLR. Thus, the lack of helper activity in newborn spleen cells is due to active suppression. Consistent with previously described neonatal suppressors of GVH [432] and contact sensitivity reactions [377], the inhibitory cell for MLR was found in the newborn spleen, but not in the thymus [23]. The inhibitory activity on neonatal spleen was unaffected by removal of glass-adherent cells, but was eliminated by removal of Thy-1$^+$ cells, indicating that the suppressor cell is a T cell.

Further studies showed that in contrast to the findings of *Pavia and Stites* [356], the neonatal spleen suppressor T cell effectively inhibited both cell proliferation and the generation of cytotoxic cells in mixed lymphocyte cultures [24]. The suppressor activity is inversely correlated to the MLC reactivity in the spleen of young animals. Thus, spleens from mice up to 8 days of age have low MLR and strong suppressor activity. From 11 to 16 days of age spleens still have relatively low MLR, but they also have low suppressor effects. By 19 days of age there is full adult-like MLR and no suppressor activity is detectable.

Rollwagen and Stutman [402] have also observed a parallelism between the maturation of culture-generated suppressor functions in ontogeny and the immune functions they regulate in vitro. It is established that T cells with proliferative capacity to alloantigens are present at birth, whereas alloreactive cytotoxic cells appear later [367, 498]. Adult lymphocytes cultured in the absence of antigen for several days develop the ability to suppress the proliferative and cytotoxic response of noncultured syngeneic lymphoid cells to alloantigens [326]. Newborn liver and spleen cells precultured for 3–5 days were shown to be capable of suppressing MLR of fresh adult cells to a similar degree as precultured adult cells [401, 402]. However, unlike precultured adult cells, newborn precultured cells were not able to suppress the generation of alloreactive cytotoxic cells. Culture-generated suppressor cells for adult cytotoxic T cells were obtained from spleen and liver of animals only at 1 week of age or older. The serum requirements for the generation of culture-induced suppressors for MLR and CTL differed. Cells inhibitory for the MLR could be generated in horse serum but required fetal calf serum for their expression. Suppression of CTL generation was not dependent on any particular serum supplement [401].

Newborn inhibitory T cells of both the antigen-specific and naturally occurring 'nonspecific' types which suppress cell-mediated reactions have been classified in many systems as T lymphocytes [23, 24, 199, 356, 432]. Also, it has recently become apparent that newborn spleens may contain, in addition to T cells, other suppressor cells of a non-T type [199, 392, 402]. Recent studies by *Hooper and Murgita* [199] have defined a newborn splenic B lymphocyte-like inhibitory cell for primary MLR on the basis of (a) adherence to Ig-anti-Ig columns; (b) nonadherence to plastic dishes or Sephadex G-10; (c) insensitivity to cytotoxic treatment with anti-T cell reagents, and (d) differential agglutination with lectins known to selectively interact with B or T lymphocytes. Other studies have reported on the existence of non-T newborn suppressors with macrophage-like properties [402, or with characteristics distinct from classical B, T, macrophages or natural killer cells [398].

As in the case of the antibody-producing system, there is now convincing evidence for the existence of efficient inhibitory T cells in the neonatal mouse for various cell-mediated reactions. Whether these inhibitory T cells act in an independent manner or whether they interact with other cells in the formation of regulatory circuits is not yet clear. We would favor the latter view. We would also like to stress that studies of the murine newborn spleen populations have revealed a particularly dynamic system with regard to cellular composition and functional changes with time after birth. A further understanding of this dynamic state will require particularly stringent experimental designs and use of defined conditions to allow fruitful comparisons between results obtained by different laboratories.

B. Humoral Mediators of Suppression

The number of humoral immunoregulatory factors described in recent years has been enormous. Although the explosion of information in this area of immunology practically defies description, readers can refer to recent articles by *Nelson and Gatti* [327], *Waksman and Namba* [488], *Cooperband and Badger* [104] and *Kamo and Friedman* [217] which classify many of the known soluble immunoregulatory factors. With these comprehensive reviews as a background we will select and consider only certain literature which may be deemed pertinent to immune regulation in the fetus and newborn. Some emphasis will be placed on our own particular contributions to this area, namely the immunoregulatory role of alpha-fetoprotein [311].

There have been a number of studies describing immunosuppressive factors in normal mouse serum [58, 267, 328, 484]. While the inhibitory

factors in normal serum have not been fully characterized, there is a growing list of substances found in serum which have immunosuppressive properties in vitro. These include alpha-globulins [104, 382], C-reactive protein [297 to 299], lipoproteins [87, 295], alpha$_1$-acid glycoprotein [90], alpha$_2$-H globulin [57], interferons [439], and prostaglandins [171, 172, 385]. Unfortunately, the present lack of information concerning the ontogenetic development of many of these serum constituents precludes any speculation, even on a teleological basis, as to their possible immunoregulatory roles in the fetus and newborn.

Fetal calf serum, a commonly used serum supplement for tissue culture media, is known to be capable of significantly altering lymphocyte reactivity in vitro [143, 206, 223, 224, 228, 282, 348, 354, 358, 401, 426, 461, 475, 505]. Thus, nonspecific suppressor cell activity is induced by culturing mouse lymphocytes in the presence of fetal calf serum for 3–5 days [62, 212, 223, 326, 354, 401]. Fetal calf serum-dependent culture generated suppressors have been shown to affect antibody synthesis to thymus-dependent [62, 354] and thymus-independent [62] antigens, mixed lymphocyte reactivity [326, 401], and cell-mediated cytotoxic responses [223, 326]. The inhibitory cells activated by fetal calf serum appear to be T lymphocytes in many of the systems studied [62, 212, 354]. Fetal calf serum has also been shown to induce strong cytotoxic responses in both human [505] and murine [426] lymphocyte cultures. In addition to the immunosuppressive effects observed in vitro by fetal sera of human [26], bovine [223], and murine [313] origin, amniotic fluid, another regular feature of the mammalian fetal environment, has also been shown to have inhibitory properties both in vitro [131, 207, 258, 314, 478] and in vivo [337, 434, 474]. Mouse amniotic fluid (MAF) suppresses both T cell-dependent and T cell-independent antibody responses, in addition to lipopolysaccharide-induced polyclonal antibody synthesis [131, 314]. Amniotic fluids have been shown to effectively block mitogen reactivity, but not alloantigen-induced lymphocyte proliferation [258]. Sera from newborn mice [214, 313] and rats [491] are also inhibitory in vitro for T cell-dependent antibody synthesis [241, 313], mixed lymphocyte reactivity [241, 491] and mitogen responses [241, 491]. It is certain that fetal and newborn sera, and amniotic fluids contain more than one, and probably several substances capable of altering lymphocyte function in vitro in both positive and negative manners. For example, MAF has been shown to exert both suppressing and augmenting effects on mitogen- and alloantigen-induced lymphocyte proliferation in vitro [316]. Serum transferrin, present in MAF at a concentration of 300 μg/ml, was found in its purified form, to cause significant lymphocyte stimulation [316]. In addition, part of the stimulatory activity of MAF

Table VI. Effects of low and high molecular weight components of mouse anmiotic fluid on T cell-dependent and T cell-independent antibody responses in vitro

Preparation	IgM PFC/culture ± SEM (% suppression)			
	SRBC-PFC	DNP-specific PFC		
		DNP-KLH	DNP-Ficoll	DNP-POL
NMS	1,150 ± 132	1,191 ± 121	665 ± 44	770 ± 62
MAF	330 ± 62 (71)	337 ± 95 (72)	298 ± 32 (56)	374 ± 24(52)
Dialyzed MAF	295 ± 42 (75)	320 ± 64 (73)	680 ± 36 (0)	755 ± 40(3)
PM10 retained MAF	367 ± 81 (69)	475 ± 110(61)	725 ± 108(0)	689 ± 60(11)
PM10 passed MAF	1,316 ± 169(0)	1,030 ± 185(13)	283 ± 15 (58)	204 ± 48(74)

Normal mouse serum (NMS), mouse amniotic fluid (MAF), dialyzed MAF, and the fractions of MAF which are retained (> 10,000 mol. wt.) and passed (< 10,000 mol. wt.) using an Amicon Ultrafiltration cell (Amicon Corp., Lexington, Mass.) fitted with PM-10 Diaflo membranes were added at 200 μg/ml to Marbrook cultures containing 20×10^6 CBA/H spleen cells immunized with TD (SRBC, DNP-KLH) and TI (DNP-Ficoll, DNP-POL) antigens. Specific antibody responses were measured as described in table I.

for MLR was shown to be in the low molecular weight dialyzable fraction. It has also been observed by us [314] and by others [131] that MAF exerts suppressive effects on both TD and TI antibody responses in vitro. However, as shown in table VI, the component in MAF which suppresses TI antibody synthesis is dialyzable, while a higher molecular weight moiety is clearly responsible for the MAF-mediated inhibitory effect on TD responses. Moreover, the fraction of MAF which is excluded by ultrafiltration on PM10 Diaflo membranes (which have 10,000 MW cut-off) suppresses TI, but not TD antibody responses. These findings show that MAF contains both low and high molecular weight components which are capable of altering lymphocyte functions in vitro. It is likely that a similarly complex array of immunoregulatory factors also exists in fetal, newborn, and pregnancy sera.

Fetuin, a major constituent of fetal calf serum, has been reported to have both stimulatory [176, 204, 383] and suppressive [500] effects on lymphocytes in vitro. Fetuin has been shown to stimulate the proliferation of hematopoietic stem cells in mice [236], to promote growth of bovine ovary cells in vitro [383], enhance rosette-forming human T lymphocytes [176], and to induce blast transformation and DNA synthesis of human peripheral blood lymphocytes in culture [204]. It would thus appear that in addition to serum

albumin [20, 371, 444] and transferrin [476, 477], fetuin is another serum component possessing significant growth-promoting properties for mammalian cells in vitro. Fetuin has also been reported to have immunosuppressive properties [282, 500]. For example, fetuin inhibits the ability of the isomitogenic mixture called H-PHA to induced lymphocyte transformation in a manner which can be reversed by increasing the dose of mitogen [282]. Since H-PHA can interact with fetuin and form complexes [500] it is assumed that fetuin-mediated inhibition of H-PHA responses can be attributed to competitive inhibition of the mitogen. However, it has also been shown that fetuin suppresses lymphocyte responses to L-PHA, an isomitogen which does not interact with fetuin [500]. This finding, together with other results describing the fetuin-mediated suppression of one-way-mixed lymphocyte reactivity [500], imply that fetuin may be able to exert a direct inhibitory effect on activated lymphocytes in vitro. It is notable, however, that only relatively high concentrations ranging from 1.25 to 2.50 mg/ml of commercially prepared fetuins were shown to be suppressive [500], whereas more purified fetuin was not significantly inhibitory at or below these concentrations [475]. Furthermore, commercial preparations of fetuin can be shown to be contaminated with AFP, a distinct embryonic substance [233] with proven immunosuppressive ability in vitro [311]. This would tend to leave some doubt as to whether fetuin itself possesses any significant immunosuppressive properties in vitro.

Mammalian alpha-fetoprotein, a glycoprotein of primarily hepatic origin [1, 148, 161] is a major constituent of serum and amniotic fluids during embryonic life [160, 268] and in murine species is still present in significant quantities in the newborn [347]. The levels of AFP then gradually diminish after birth to the low nanogram amounts normally present in adult serum [408, 409]. Aside from transient elevations in fetal-derived AFP which occur as a normal physiological event in the pregnant female, resynthesis of AFP in the adult usually reflects an abnormal expression of this embryo-specific protein signifying disease. Increased serum AFP levels have been found in a number of malignant and nonmalignant diseases [7] with high and sustained serum AFP concentrations being essentially diagnostic for primary liver cancer [1]. Molecules of AFP are composed of single polypeptide chains containing about 4% carbohydrate with a molecular weight of approximately 70,000 daltons [410]. Immunochemical analyses of AFPs from several different species have revealed both common and species-specific properties in their molecular structures. The similarities among the AFPs from various mammalian species are indicated by significant antigenic cross-reactions [210]. More-

over, the immunological cross-reactivity between the denatured peptides of AFP and albumin [406] support conclusions derived from sequence data [407] that these two serum proteins are structurally related and may even share a common ancestral gene. It is important to note that a number of different molecular subspecies of AFP have been detected by conventional polyacrylamide-gel electrophoresis [13, 177], extended agarose-gel electrophoresis [249], and by lectin [36, 435] and estradiol [482] affinity chromatography. Results in the murine system show that the increase in sialytransferase activity in fetal liver with time of embryonic development correlates with the increased proportion of sialylated molecular variants of AFP [178, 506]. However, molecular variants of AFP have been shown to be partly, but by no means solely, due to variation in degree of sialylation [249, 250]. The biochemical basis of the extensive microheterogeneity remaining after desialylation is unknown.

AFP has thus been a well-studied 'onco-fetal' substance with regard to its physical-chemical properties, its normal and pathological distribution, and its diagnostic significance. However, until recently, very little had been learned about the possible biological functions of AFP. There is some evidence that AFP may function as an estrogen-binding receptor protein involved in protection of fetal tissues against circulating maternal estrogens [483]. However, strong estrogen-binding capacity appears restricted to AFPs of certain animal species [335] perhaps due to the presence of varying proportions of molecular subspecies of AFP with different estrophilic properties. It is therefore likely that AFP has other important functions in addition to its hormone-binding capacity. We have, for some time, been exploring the premise that AFP may play an important immunoregulatory role during ontogeny. The normal distribution of AFP during ontogenetic development would be consistent with such a function. Thus, in the murine system the physiological postnatal decline of serum AFP levels [347] is seen to correlate with the onset of adult-like immune reactivity.

A clear demonstration that highly purified AFP could suppress certain immune functions was initially obtained in the murine system [315, 316]. Mouse fetal-derived AFP was shown to exert a potent noncytotoxic inhibitory effect on primary IgM and secondary IgM, IgG and IgA anti-SRBC PFC responses in vitro [315]. Significant inhibition of TD primary antibody synthesis occurred with concentrations of AFP as low as 1.0 μg/ml. Dose-response studies revealed a differential inhibitory effect by AFP on the various immunoglobulin classes of antibody with IgA being most sensitive to suppression, IgG being moderately affected, and IgM responses the least

susceptible [315]. Lymphocyte proliferation induced by mitogens and alloantigens was also shown to be prone to AFP-mediated suppression [316]. Thus, AFP in concentrations comparable to those shown to suppress antibody synthesis [315] effectively inhibites normal splenic lymphocyte proliferation by the T-cell mitogens Con A and PHA, the presumed B cell mitogen LPS, and mitomycin-treated allogeneic spleen cells in a one-way mixed lymphocyte reaction [316]. Studies by *Zimmerman* et al. [507] have confirmed that murine AFP isolated from amniotic fluid or fetal plasma is suppressive for in vitro anti-SRBC antibody synthesis. They further showed that the presence of sialic acid residues in the AFP molecule was necessary for immunosuppression. *Sheppard* et al. [423] demonstrated that AFP isolated from sera of hepatoma-bearing mice as well as amniotic fluid diminished primary in vitro antibody synthesis and reduced by 2-fold secondary antibody responses. Rat AFP of fetal [421] but not tumor [355, 421], origin was shown to suppress certain T cell reactions in vitro.

It would appear from our studies that the suppressive ability of AFP is preferentially expressed on the T cell-dependent limb of the immune response. Thus, IgA antibody synthesis which is considered highly dependent on thymus-derived T cells [98, 262] is the most sensitive to AFP-induced suppression, while IgM responses are least affected by AFP, and are also thought to be relatively T-cell independent. T cells which regulate B cell proliferative responses to LPS [333] are the likely targets for AFP-mediated suppression of LPS responses [316]. As pointed out earlier in this article, newborn mice can synthesize antibodies to many TI antigens within a few days after birth, whereas full antibody responsiveness to TD antigens is delayed for 4–6 weeks. If endogenous AFP does function in vivo to regulate antibody synthesis in the newborn mouse, then one would predict that TD antibody responses would be more susceptible than TI responses to AFP-mediated suppression. We therefore performed experiments to compare the inhibitory effects of AFP on in vitro antibody responses to the TD antigens, SRBC and DNP-KLH versus the TI antigens, DNP-POL and DNP-Ficoll [311, 314]. Shown in table VII are the results of a representative experiment demonstrating that AFP does effectively suppress specific antibody responses to TD antigens, but fails to significantly affect TI antigens. Polyclonal B cell antibody synthesis stimulated by LPS was also found to be unaffected by AFP [314]. These findings tend to exclude a direct inhibitory effect by AFP on B cell functions or on macrophages required for TI antibody synthesis [92, 247], and provide further evidence that T cells are a primary target for AFP-mediated immunosuppression. The additional find-

Table VII. Effect of AFP on T cell-dependent versus T cell-independent antibody synthesis in vitro

Preparation	Immunizing antigen	IgM PFC/culture ± SEM (% suppression)		
		SRBC-PFC	DNP-specific PFC	
			SRBC-TNP_4	SRBC-TNP_{40}
NMS	SRBC	1,33 ± 88		
AFP	SRBC	170 ± 69(85)		
NMS	DNP-KLH		404 ± 76	1,004 ± 96
AFP	DNP-KLH		97 ± 14(76)	321 ± 58(68)
NMS	DNP-Ficoll		413 ± 29	893 ± 55
AFP	DNP-Ficoll		432 ± 38(0)	799 ± 24(11)
NMS	DNP-POL		168 ± 18	417 ± 34
AFP	DNP-POL		211 ± 69(0)	434 ± 42(0)

Normal mouse serum (NMS), and fetal-derived mouse alpha-fetoprotein (AFP) purified according to procedures described in detail elsewhere [315] were added at 200 μg/ml to Marbrook cultures containing 15×10^6 CBA/J spleen cells and optimal immunizing doses of TD and TI antigens (see table I). DNP-specific PFC were detected using TNP coupled SRBC as indicator cells. TNP groups were coupled to SRBC in low (SRBC-TNP_4) and high (SRBC-TNP_{40}) epitope densities by mixing 4 mg and 40 mg of 2,4,6-trinitrobenzen-sulfonic acid (Sigma Chemical Co.) per ml of packed SRBC [for details see reference 314].

ing in this study [314] that PHA responses were more effectively suppressed by AFP than were Con A responses, offered presumptive evidence for preferential action on certain T cell subpopulations as defined by these two T cell mitogens [455].

The selective nature of the in vitro immunoregulatory effects of AFP was further revealed in a series of experiments testing the impact of AFP on T cell proliferation towards defined histocompatibility-associated alloantigens [320, 359, 494]. In these studies the effect of AFP was tested on the four major genetic systems known to induce strong MLR activation in the mouse [360]: the Ia antigens, the serologically defined (SD) products of the major histocompatibility complex (MHC) K or D regions, the Mls locus products, and the products of an as yet undefined non-MHC system associated with a limited number of strains (i.e. DBA/2, B10.D2). The addition of AFP to mixed lymphocyte cultures at intiation could be shown to almost completely eliminate cell proliferation against Ia and/or M locus structures, while

normal numbers of T blasts were observed when stimulations were carried out against SD or non-MHC loci determinants [359]. It was possible to conclude from these findings that AFP exerts its suppressive activity on MLR through a selective interference with I region triggering systems. Moreover, AFP will not only usually fail to inhibit I region independent lymphocyte-activating systems, but actually will sometimes substantially augment these reactions. Subsequent analysis of the effect of AFP on cell-mediated lympholysis (CML) showed that the genetic relationship between responding and stimulating cells also largely predetermines whether or not AFP will inhibit the generation of CTLs [312, 361]. Thus, effector cells generated during lymphocyte activation with certain stimulator strains including DBA/2 and B10.G followed by testing for cytolysis on P815 tumor [312] or LPS-stimulated spleen cell targets [361] were found to be insensitive to AFP-mediated suppression. It is notable here that lymphocyte proliferation induced by non-MHC alloantigen systems on DBA/2 cells is not suppressed by AFP [359]. However, AFP did exert strong suppressive effects on CTL generation when using strain combinations with genetic differences in individual SD regions of the MHC [361]. The inhibition occurred independently of the effect of AFP on the MLC-activating phase, as normal numbers of T cells responded via cell division when confronted with SD antigens in the presence of AFP, but there was no subsequent generation of cytolytic T cells [361]. Since there is evidence for a necessary T-T cell collaboration between Lyt 1 helper cells responding against LD determinants and Lyt 2 CTL precursors reacting to SD determinants for effective generation of CTLs, it seems likely that AFP is blocking at the Lyt 1 helper cell level. If alloantigenic differences exist both with regard to SD and non-MHC, then there will be a normal generation of killer T cells against SD determinants in the presence of AFP [361]. These findings indicate that lymphocyte activating, non-MHC determinants can circumvent the mechanism of AFP-induced inhibition, presumably by supplying a non suppressible helper T cell signal to the 'poised CTL' reacting against SD. This contention is supported by the findings of *Schendel and Bach* [416] which indicate that lymphocyte activation by non-MHC determinants can indeed provide the helper T cell signal to CTL precursor cells.

It is clear from the observations outlined above that AFP will not serve as a general inhibitory agent for all immunological reactions. On the contrary, AFP appears to regulate TD antibody synthesis and certain cell-mediated reactions in a highly selective manner. A primary target for AFP-mediated suppresion would appear to be an Lyt 1^+ T helper cell required for

antibody responses to TD antigens and for LD-SD collaboration with pre-killer cells in the cellular events leading to optimal CTL generation [15, 28]. Data obtained by other workers failing to obtain significant inhibition of certain murine [190, 423] or rat [355, 421] immune reactions in the presence of AFP should be more easily understood when the above knowledge as to cellular and genetic restrictions is taken into account.

There have been a number of reports demonstrating that human AFP is capable of suppressing certain types of lymphocyte functions in vitro [25, 175, 251, 253, 321, 501]. *Yachnin and Lester* [501] have shown that AFP isolated from human fetal liver is a more potent suppressor of mitogen- and alloantigen-induced lymphocyte transformation by 1–3 orders of magnitude over tumor-derived AFP. Subsequent studies by this group [251] showed a correlation between the relative amount of a particular negatively charged molecular variant of AFP and immunosuppressive strength. Thus, fetal liver derived AFP, which has a high content of the electronegative species, is strongly immunosuppressive, while the usually weaker inhibitory properties of various tumor AFP isolates can be linked to the relative amounts of the negatively charged molecular variant they contain [251]. In view of the highly selective nature of the immunosuppressive action of murine AFP, we undertook an investigation to dedermine whether human AFP might also exert selective inhibitory effects on B and T cell proliferative responses in vitro [321]. The effects of human fetal-derived AFP were compared on lymphocyte activation induced by the human B cell mitogen protein A of *Staphylococcus aureus* strain Cowan I [144], by the T cell mitogen PHA, and by irradiated allogeneic lymphocytes in the one-way mixed lymphocyte reaction. Mitogenic responses to PHA were strongly inhibited in a dose-dependent manner over an AFP concentration range of 300–18 μg/ml. Importantly, PHA-reactive T cells were shown to be inhibited by a mechanism other than binding interference or competition with mitogen for cell surface receptors. In parallel cultures the proliferative response to protein A expressing *S. aureus* was normal or slightly enhanced in the presence of AFP. Strong suppression of the MLR generally requires higher concentrations of AFP than were necessary for inhibiting PHA responses [321]. The relative insensitivity of human MLR to AFP-mediated suppression, in comparison to PHA responses, may be an indication that, as in the murine system, human AFP is capable of exerting selective inhibitory effects on certain T cell subclasses and/or that the inhibitory action of human AFP on allogeneic reactions is restricted by the genetic relationship between responding and stimulating cells. Evidence does exist for functionally distinct subpopulations of human

T cells [86], and several reports indicate that mitogens and alloantigens may activate separate T cell subpopulations [18, 454]. Studies by *Gupta and Good* [175] and *Littman* et al. [259] would also suggest that human AFP is selective in its suppressive effects on in vitro cellular immune responses. Moreover, there are reports of AFP-mediated augmentation of mitogen and alloantigen-stimulated lymphocyte proliferation [85, 321, 441]. We consider that the immunoenhancing effects of AFP may be a physiologically relevant phenomenon. A growth-promoting effect by AFP on distinct cell types would be consistent with its frequent association with rapidly dividing cell populations in the fetus and newborn, and may to some extent reflect a shared property with albumin, a closely related protein thought to be essential for growth of mammalian lymphocytes in vitro. It should also be noted that unlike our experience with murine AFP we [321] and others [501] have observed a wide variation in the suppressive effects among individual isolates of human AFP. The exact reasons for these variations are not clear, but differences in methodology employed to isolate [64] and test [321] the proteins, the fetal versus tumor origin of the protein [501], the existence of a unique immunosuppressive subspecies [251] which may undergo postsynthetic modification [252], and the loss of active moieties during purification which are normally bound to AFP in the native state [226] are all possible contributing factors. Only further detailed investigations will determine which, if any, of these variable factors can satisfactorily explain presumed inconsistencies in the literature. Nevertheless, on the basis of the large body of information presently available, it is possible to conclude with a high degree of certainty that some molecular species of AFP do indeed possess an intrinsic capacity of mediate highly selective and powerful immunoregulatory influences on immune systems in vitro. AFP molecules with intrinsic suppressive properties may, however, also act in conjunction with other active factors in complicated immunoregulatory pathways.

We have studied the mechanism of AFP-mediated suppression on in vitro antibody synthesis in the murine system [167, 322]. Experiments were performed to determine whether appropriate in vitro exposure of normal adult spleen cells to AFP would result in the activation of a population of 'suppressor cells'. In initial studies adult spleen cells were cultured in Marbrook chambers [266] in the presence of AFP or normal mouse serum (NMS) at a final concentration of 200 μg/ml for 4 days. The precultured cells were then washed and 1×10^6 cells were transferred to secondary assay cultures consisting of 20×10^6 fresh normal syngeneic spleen cells along with optimal immunizing doses of TD antigen. After an additional 4 days of

incubation the assay cultures were harvested and IgM antibody synthesis was measured. Spleen cells precultured in AFP were shown to effectively suppress primary IgM antibody responses, while cells precultured in NMS did not [322]. The fact that suppressor activity was significantly enriched in precultures of purified splenic T cells in comparison to whole spleen precultures, and that suppressor activity was absent in precultures of nude mouse spleen [322] indicated that the AFP-induced inhibitory cells were T lymphocytes. Interestingly, these adult AFP-activated inhibitory T cells showed many similarities with the previously mentioned naturally occurring newborn suppressors (table I) in terms of cell type, function, and efficiency of suppression. Like the newborn inhibitory T cells, adult AFP-activated T lymphocytes were found to suppress TD, but not TI antibody synthesis [318, 322].

Dilution analysis showed that as few as 10^4 adult inhibitory T cells, representing 0.05% of the total assay cilture cell population markedly inhibited anti-SRBC responses [322]. In view of the functional similarities between newborn and adult inhibitory T cells we sought to determine whether the two cell populations might have common cell surface differentiation antigen phenotypes. Table VIII shows the effects of cytotoxic pretreatment of AFP-activated adult inhibitory T cells with anti-T cell reagents on their subsequent ability to inhibit TD antibody synthesis in secondary assay cultures. Treatment with heterologous anti-T or anti-Thy-1 antibodies and complement eliminates the ability to initiate suppression. Likewise, cytotoxic pretreatment with anti-Lyt 1 antibody abolished the suppressing activity, while anti-Lyt 2 failed to impair the inhibitory effect. These results therefore show that, like naturally occurring newborn suppressor (table II), the AFP-induced inhibitory cells in adult spleens also belong to the Lyt 1^+2^- subset of T lymphocytes. Additional studies were performed to determine the cell surface Ia antigen phenotype of adult AFP-activated inhibitory T cells [200]. The results of a typical experiment are presented in table IX. It can be seen that the suppressor activity was abrogated by cytotoxic pretreatment with anti-H-2^k, anti-Ia^k, anti-I-ABJ^k and anti-I-J^k antisera, but not by anti-I-EC^k. Only antisera which contained anti-I-J^k reactivity were effective in removing the suppressing ability thereby adding I-J coded determinants to the already known existence of Lyt 1 determinants on adult AFP-activated inhibitory cells. Thus, adult splenic T cells activated by a 4-day preculture in the presence of AFP could be shown to display the very same functional properties and cell surface antigen phenotype (Thy-1^+, Lyt 1^+2^-, I-J^+) as the naturally occurring newborn suppressing cells. Moreover, the rate of dis-

Table VIII. Cell surface Lyt differentiation antigen phenotype of AFP-induced adult inhibitory T cells

Preculture	Cytotoxic treatment with antisera and complement	Assay culture, % of control PFC response (mean ± SEM of 5 experiments)
–	–	104 ± 4
T_{NMS}	–	49 ± 3
T_{AFP}	–	50 ± 2
T_{AFP}	+ RC	92 ± 5
T_{AFP}	anti-T + RC	84 ± 4
T_{AFP}	anti-Thy-1.2 + RC	90 ± 4
T_{AFP}	anti-Ly 1 + RC	47 ± 3
T_{AFP}	anti-Ly 2 + RC	

20×10^6 Ig-anti-Ig column purified adult CBA/J spleen T cells were precultured for 4 days in Marbrook chambers in the presence of 200 μg/ml normal mouse serum (NMS) or alpha-fetoprotein (AFP) [see references 318 and 322 for details]. Precultured T cells were washed, treated with antisera plus rabbit complement (RC), and aliquotes equivalent to 10^6 control untreated cells were tested for inhibitory activity on IgM anti-SRBC PFC responses in secondary assay cultures. The number of day-4 IgM anti-SRBC PFC in control cultures without added precultured T cells was 832 ± 57.

appearance of inhibitory T cells from the spleens of newborn mice with increasing age is closely paralleled by a descrease in the levels of serum AFP [318]. On the basis of these findings we would conclude that endogenous AFP may play an important immunoregulatory role in the developing immune system of the fetus and newborn via the induction of such inhibitory T lymphocytes. In support of this contention are reports that both newborn and adult AFP-activated inhibitory cells can function in vivo to suppress IgG and IgA antibody synthesis [200], and that adult mice injected with AFP have heightened susceptability to Moloney sarcoma virus oncogenesis due to AFP-induced suppressing T lymphocytes [157]. In the human system we [322] and others [14] have shown that purified human AFP can activate in vitro cultured T cells isolated from normal peripheral blood to become inhibitors of mixed lymphocyte reactivity. The results presented in table X demonstrate that normal peripheral blood T lymphocytes exposed in vitro to purified fetal-derived AFP for 4 days can also effectively suppress PHA-stimulated lymphocyte transformation. The controls for this experiment consisting of T cells precultured in an equivalent concentration of human fetal-derived albumin failed to induce any inhibitory activity for mitogen

Table IX. Presence of I-J determinants on AFP-induced adult CBA/J inhibitory T cells

Preculture	Treatment										Assay culture
	antiserum + RC	H-2 region detected									I_gM SRBC PFC/ culture ± SEM (% suppression)
		K	I-A	I-B	I-J	I-E	I-C	S	G	D	
–	–										668 ± 99
T_{NMS}	–										716 ± 96(0)
T_{AFP}	–										324 ± 36(51)
T_{AFP}	RC										234 ± 57(65)
T_{AFP}	anti-T										652 ± 41(2)
T_{AFP}	anti-Thy 1										656 ± 81(2)
T_{AFP}	C3H. SW anti-C3H	K	K	K	K	K	K	K	K	K	636 ± 20(5)
T_{AFP}	A. TH anti-A. TL		K	K	K	K	K	K	K		680 ± 62(0)
T_{AFP}	(A. THXBIO. HTT) F_1 anti-A. TL		K	K	K						764 ± 70(0)
T_{AFP}	BIO. A (3R) anti-BIO. A (5R)				K						868 ± 62(0)
T_{AFP}	BIO. S (7R) anti-BIO. HTT					K	K	K	K		277 ± 24(58)

Adult AFP-activated inhibitory T cells (T_{AFP}) were generated in vitro according to procedures described in detail elsewhere [see table VIII, and references 318 and 322]. The Ia phenotype of T_{AFP} was determined as described for newborn inhibitory T cells in table III.

Table X. Human AFP-induced suppressor T cells: demonstration on PHA response

Preculture		^{3}H-TdR incorporation, cmp (mean ± SE)	
T cells precultured in	number of precultured cells added to assay culture	assay culture PHA 1.0 μg/ml	assay culture PHA 0.1 μg/ml
–	–	57,354 ± 1,259	26,321 ± 2,031
Albumin	10^5	51,540 ± 2,468	20,475 ± 976
AFP	10^5	37,027 ± 2,930	11,825 ± 1,454
Albumin	10^4	58,164 ± 1,115	21,065 ± 1,025
AFP	10^4	39,320 ± 1,544	10,667 ± 1,804

Normal human peripheral blood T lymphocytes, isolated on Ig-anti-Ig columns, were precultured at 8×10^6 per ml in Marbrook chambers for 4 days in the presence of purified human fetal-derived AFP or albumin at final concentrations of 200 μg/ml. 1×10^5 precultured T cells were transferred to assay cultures consisting of 2×10^5 syngeneic peripheral blood lymphocytes and PHA. Mitogen responses were measured at 72 h of culture.

responses. Indeed, we have consistently observed that purified human fetal albumin lacks suppressive activity when added directly to mitogen- and allo-antigen-stimulated lymphocyte cultures [321], or to precultures to test for induction of inhibitory cells for MLR [311]. On the basis of the above-mentioned findings and our previously discussed data in the murine system, one could speculate that the AFP present in cord blood may function as an inducer of the human newborn suppressor T cells described by *Olding* et al. [341–344].

In addition to the indirect mode of immunosuppressive action by AFP via the induction of regulatory suppressor cells, we have obtained evidence which suggests that AFP can also exert direct antiproliferative effects on select populations of MLC-reactive T cells [359]. Thus, in MLR studies we [359] have shown that addition of murine AFP to isolated anti-Ia T blasts responding in secondary MLC results in an almost immediate block of ^{3}H-thymidine incorporation following restimulation. The question of whether this effect is related to the cell-binding properties of AFP [33, 109, 462] remains to be determined. Extensive studies in both the murine and human systems would therefore indicate that AFP, in its purified form, will suppress either directly or indirectly certain T cell-mediated immune reactions while leaving others unaffected or sometimes enhanced. Existing evi-

dence suggests that AFP does not act to affect B cell functions in a direct manner. However, there is evidence that AFP can exert suppressive effects on other cell types, including macrophages [207, 346], and estrogen-sensitive tumor cells [440] implying a more general biological significance for the regulatory properties of AFP. A recent series of findings indicate that AFP may be also important in the regulation of autoimmune responses. Human AFP has been reported to inhibit the binding of myasthenia gravis antibody to acetylcholine receptor antigen in vitro [5, 56]. Interestingly, the authors suggest that the existence of AFP in the fetus and newborn may explain the observation that transitory neonatal myasthenia gravis occurs only in a minority of cases in spite of the presence of transferred maternal anti-acetylcholine receptor antibodies. Additional preliminary reports suggest that the development of experimental autoimmune myasthenia gravis [55] and allergic encephalomyelitis (EAE) [4] can be prevented in animals by appropriate pretreatment with injections of AFP. In a separate study [147] it was noted, however, that while newborn rats with high endogeneous levels of AFP do have a low incidence of EAE, a close correlation between decreasing serum levels of AFP and increased susceptibility to EAE is not observed. *Fujinami* et al. [147] therefore concluded that the presence of AFP does not explain the lack of disease in newborn rats. Finally, there is strong evidence emerging in the murine system which supports the contention that AFP may play a very important role in the prevention of autosensitization [*Hooper and Murgita*, 'Alpha-fetoprotein suppresses autoreactive T cell proliferation in vitro', submitted for publication]. In this study we have shown that neonatal or adult murine Lyt 1^+ T cells reacting in autologous mixed lymphocyte reactions are suppressed in a highly manner by purified fetal-derived AFP in concentrations that are well below the normal endogenous serum levels in the fetus and newborn. As we have pointed out earlier [311], the possible role of immunoregulatory AFP in the clinical course of autoimmune disease is not yet clear. Nevertheless, the above-mentioned findings with their obvious potential theoretical and clinical importance should serve to stimulate further investigations in this area.

C. Regulation of Natural Killer Cell Activity

Natural killer (NK) cells constitute a distinct group of cells not belonging to any of the 'classical' types of cells in the immune system [194, 231]. They have recently attracted great interest due to their lytic effect on certain malignant cells in vitro. There is also a positive correlation between the level of NK activity in the murine system and the in vivo resistance to certain

tumor types [229]. In addition to their capacity to kill tumor cells, NK cells have been found to have lytic reactivity towards certain bone marrow stem cells [*Hansson* et al. 185a] as well as against immature, cortical thymocytes [184]. NK cells would therefore seem to be endowed with a special ability to kill certain cells expressing embryonic or 'primitive' features [449]. As most targets for NK cells include stem cells in the bone marrow as well as immature thymocytes it is likely that natural killer cells would also express some important features in relation to the neonatal period. Since this is clearly true for the rodents we include here a summary of the results of NK activity in relation to age.

A characteristic feature of the NK system in mice and rats is the rapid rise and fall in NK activity with age [193, 230]. Thus, NK reactivity is largely absent in fetal liver cells and in the spleens of newborn mice but will show a rapid rate of appearance at around 3 weeks of age. Activity will peak at 6–8 weeks of age and will then decline. A similar although not as abrupt pattern is displayed in the rat. Human NK cell activity shows a less impressive correlation to age although an age impact is clearly noted [67]. There may be several underlying reasons why NK cells seem to be comparatively more completely 'suppressed' in the newborn mouse than several other cellular types. One possibility would be that these cells are physically absent in the very young animals. In support of such a view are the findings that spleen cells from young mice (2 weeks old) possess only about half the number of NK cells as measured in a target-binding assay [*Roder*, personal commun.] as are in young adult mice. However, considering the relatively high amounts of erythropoietic cells in newborn spleen compared to adult [492], dilution effects must seriously be considered. Another plausible reason for the low level of NK activity noted in newborn mice may reside at the level of regulatory 'suppressing' cells. Thus, it has been known for some time in adult mice that the organ distribution of NK activity is inversely related to the actual presence of 'natural' target for NK activities in the same organ [229]. Thus, thymus and bone marrow which constitute two organ systems quite low in NK activity are also the two organs with the highest numbers of cells that can be lysed *in vitro* by NK cells [184, 185]. The frequency of such naturally occurring NK targets is significantly increased in the fetal and newborn periods both in murine and human systems. These target cells can be shown to be able to function as efficient cold target inhibitors in NK cytolytic assays [184, 185]. It would therefore follow that the presence of such potential target cells in a given organ would serve to reduce (i.e. suppress) the

actual available NK cells in the same population. As this 'cold target' inhibition would be expected to also involve conjugate formation between NK cells and target cells in vivo this would automatically lower the number of NK cells available for the conventional target conjugate assay in vitro and thus yield false low values. Finally, a third possible reason why newborn mice have close to undetectable levels of NK cells may be at the level of endogenous regulation of interferon levels [284, 422]. The newborn mouse does seem to have somewhat altered macrophage activities [260]. Polyinosinic-polycytidylic acid (poly I:C), an agent known to function via macrophages to induce NK cells through interferon release has been found by us to be an inefficient inducer when using spleen cells from newborn mice [349, 350]. On the other hand, the direct addition of interferon to newborn mouse spleen cells could induce a low but significant degree of NK activity. As interferon is known to be the major regulator of NK activity [158, 159], we consider it likely that the newborn mouse may be particularly deficient with regard to macrophage-mediated interferon production. If this mode of interferon release is an important factor in maintaining normal levels of NK activity (for which there is some precedent) [94] this may well add to the inefficient milieu with regard to the creation of sizable numbers of functioning NK cells in newborn mice. It is interesting to note that serum from newborn, but not adult mice inhibit in vitro NK activity [77]. Our findings suggest that fetal-derived AFP may in this regard play a role as poly I-C will fail to induce efficient NK activation in adult spleen cells if AFP is present in physiological amounts [349, 350]. Trivial reasons for this suppressing activity of AFP could be excluded and interferon added to adult spleen cells in the presence of AFP did yield, as expected, a significant increase in NK activity [350a].

In summary, we would thus conclude that the very low NK activities observed particularly in newborn rodents can be explained as a result of several factors all interacting to yield an 'NK-suppressing' milieu. As NK cells would seem to be able to kill certain stem cells in the bone marrow one could argue that such low NK activity at this stage may have some beneficial values for the individual.

VI. Pregnancy-Associated Immunoregulatory Activity

A most remarkable feature of mammalian pregnancy is the existence of what appear to be several precisely coordinated physiological processes

which together account for the protection of the histoincompatible fetus from maternal immune attack. Thus, attempts to define the mechanisms responsible for the successful maternal-fetal immunological co-existence have led to a number of concepts including (a) effective cellular and non-cellular anatomical separations at the maternal-fetal interphase; (b) hypo-antigenicity of the fetal-placental unit; (c) interruption of potentially harmful maternal antifetal cell-mediated immune responses via specific blocking factors (i. e. antibody-mediated tolerance-enhancement), and (d) transient hyporeactivity of the maternal immune system. Accumulated experimental evidence suggests that each of these processes may be playing a contributory role [17, 37–40, 107, 126, 191, 399]. However, while there does seem to be general agreement that exemption of the fetus from damaging maternal immune reactions is at least in part the result of the combined effects of several processes, there is no consensus as to the relative importance of each in maintaining this delicately balanced immunological liaison. A general discussion of the complex immunobiology of the maternal-fetal relationship can be found in recent review articles [37, 40, 399]. We will confine our remarks here to include only a brief discussion on the status of the maternal immune system during pregnancy. The question of whether immune functions remain unchanged, or become hypo- or hyperreactive during pregnancy is, within the context of the present article, to be considered quite relevant. For example, as pointed out by *Beer* et al. [39] one cannot completely disassociate the developing immune system of the fetus and newborn from that of the mother since there is known to be a significant level of maternal-to-perinatal transfer of serum proteins (including immunoglobulins) and milk leukocytes. Such an exchange of active humoral and cellular components is likely to have profound effects on the ontogenetic development of immune reactivity in the fetus and newborn.

There is now abundant evidence to show that the pregnant female normally does recognize and react against fetal antigens [37]. The failure of such sensitization to prejudice the fetus has been attributed to the presence of specific serum-blocking factors (thought to be antibodies and/or antigen-antibody complexes) which can be shown to effectively prevent the expression of maternal cell-mediated immunity in vitro [192, 502]. According to this premise some form of active response by the pregnant female toward the fetus would therefore be advantageous, if not essential, to the survival of the fetal 'allograft'. However, it is also quite possible that certain selective depressions of maternal immune reactivity should have to occur during pregnancy representing an additional beneficial adaptive response to protect

the fetus. Such alterations in the immune responsiveness of the pregnant female could condeivably exist at several different levels. There is, for example, evidence that certain maternal lymphocyte functions may be suppressed at intrinsic [45, 203, 265, 289, 384], extrinsic [47, 189, 219, 248, 357], local [19, 35, 146, 428] and systemic [16] levels. Pregnancy-associated immunosuppression is reported to be both specific [51, 195, 436, 437, 473] and nonspecific [30, 44, 179, 274–276], and to be mediated by humoral factors [332, 418, 452, 453], and by cells [46, 83, 84, 93, 463].

A number of physiological changes associated with the immune system are known to occur during pregnancy. It seems quiet well established, for example, that the thymus undergoes transient involution during pregnancy in mice, rats, and humans [30, 95, 399]. There are also reports of splenic hyperplasia [10] and increased numbers of T cells in the lymph nodes draining the uterus [19, 201]. Information concerning pregnancy-associated changes in circulating lymphocyte populations appears somewhat controversial. Thus, there are reports suggesting that the percentage and absolute numbers of T cells are decreased during pregnancy [60], that B cell numbers decline while T cells remain unchanged [91], that the percentage of T cells temporarily drops together with an increase in B lymphocytes [459], and that the frequencies of B and T cells do not vary significantly throughout pregnancy [31]. It may be possible to reconcile some of these findings by considering that subtle redistributions in lymphocyte subpopulations are continuously occurring at various stages of pregnancy perhaps in response to changing hormonal levels [91, 415].

Evidence that immune reactivity is lowered during pregnancy includes altered survival of allografts [195, 279, 399], and suppression of mitogen responses [16, 35, 146, 203, 384], cell-mediated cytotoxicity [437, 473], NK activity [29], host versus graft reactions [93], antibody synthesis [30, 283, 313], and macrophage function [364]. Systemic suppression appears to be weak [16] and some immune functions are clearly not intrinsically inhibited at all during the gestational period [78, 79]. Circulating pregnancy-associated humoral factors may therefore be playing important extrinsic immunoregulatory roles by providing effective local immunosuppression. A number of studies have documented the in vitro immunosuppressive effects of pregnancy sera [139, 219, 220, 248]. Attemps to isolate and characterize the active factors in sera collected at different stages of pregnancy have led to varying degrees of success. Pregnancy-associated alpha-globulins have been shown to inhibit a variety of in vitro lymphocyte functions, including mitogen- and alloantigen-induced T cell proliferation [418, 452,

453]. Interestingly, *Stimson* [452] has shown that a high molecular weight glycoprotein isolated from pregnancy serum called alpha-macroglobulin shows some degree of selectivity in its in vitro immunosuppressive action. Thus, T cell stimulation induced by Con A, PHA, allogeneic cells, and tuberculin was found to be effectively blocked by physiological concentrations of alpha-macroglobulin. On the other hand, B cell proliferation induced by LPS and by anti-$F(ab)_2$ serum was shown to be less affected by the pregnancy-associated alpha-globulin suggesting that T cells are a primary target in this inhibitory pathway. This premise was supported by a subsequent study showing that alpha-macroglobulin binds to subpopulations of T lymphocytes and monocytes, but not to B cells [451]. The possible role of immunosuppressive AFP molecules in pregnancy sera has also been investigated [139, 313, 474]. A study in the murine system has shown that there are selective depressions in certain immune functions during pregnancy which may in part be attributable to circulating levels of AFP [313]. Pregnancy induced inhibition of in vitro and in vivo specific antibody synthesis was most pronounced on the IgA and IgG classes with little or no significant effect on IgM antibody production observable in vivo [131]. It is notable here that this same pattern of selective inhibition of antibody production was previously seen in mice receiving injections from birth through young adulthood with homologous amniotic fluid [337] and in studies on the effect of purified fetal-derived AFP on primary and secondary antibody responses in vitro [315]. Spleen cells from mid-term pregnant mice had suppressed mitogenic responses to PHA, a slightly reduced response to Con A and a normal response to LPS. Allogeneic reactivity of splenic T lymphocytes from pregnant animals was significantly enhanced over non-pregnant controls. However, pregnancy sera were effective in blocking in vitro TD antibody synthesis as well as T mitogen and alloantigen-induced lymphocyte proliferation. Much of this inhibitory effect could be abrogated by selective removal of AFP on antibody-agarose affinity columns. Finally, normal mouse serum became similarly immunosuppressive in vitro when purified AFP of fetal origin was added to it in concentrations approximating the levels present in the sera of mid-term pregnant mice [313]. Studies by *Toder* et al. [474] suggest that AFP-mediated immunosuppression can be observed even in the early stages of murine pregnancy if the circulating levels of AFP are artificially boosted by injections of purified AFP. In addition, correlative studies in pregnant women between lymphocyte functions and serum AFP levels are compatible with the idea that AFP may be one among a number of factors contributing to pegnancy-induced immunosuppression [139].

Immunoprotection of the fetus could also be provided in part by the regulatory influences of the endocrine milieu of pregnancy [10]. There have been reports which suggest that the human chorionic gonadotropin (HCG) hormone which is synthesized early in pregancy by the trophoblast, may have immunosuppressive properties [6, 103, 373]. However, it is not clear from subsequent studies whether the inhibitory activity found associated with commercially prepared HCG extracts from pregnancy urine was actually due to the hormone or to a co-purified immunoregulatory contaminating substance(s) [296, 309]. There would seem to be more substantial evidence that some of the steroid hormones may possess physiologically relevant immunosuppressive activity [3, 393, 499]. Studies by *Siiteri* et al. [428] indicate, for example, that effective local concentrations of progesterone may exert significant suppressive effects on maternal T cell-mediated immune functions. Cortisol was also found to be an effective inhibitor of human MLR, although *Kasakura* [220] has shown that the inhibitory activity in pregnancy sera does not correlate with serum cortisol levels.

There is now a growing opinion that certain gestational hormones as well as some other pregnancy-associated substances, are most likely able to exert strong local immune suppression rather than mediating broad systemic immunoprotection. It is possible that the various active pregnancy-associated factors which have been described act in concert to impart beneficial immunoregulatory effects at the maternal-fetal interphase. An immunoregulatory role for pregnancy plasma factors may also be indicated by studies demonstrating the abortagenic and immunotherapeutic effects of antibodies directed against such substances [285, 286, 433]. The mechanism of suppression by pregnancy related factors could involve direct inhibitory action on relevant target lymphocyte functions and/or an indirect regulatory effect via the induction of suppressor cells. Indeed, there is now some evidence to suggest that specific [83, 84, 93] and nonspecific [46, 463] inhibitory cells do exist during pregnancy.

VII. General Conclusions

The immune response at the cellular level respresents an intricate system of inductive and regulatory cell circuits. An analysis of the ontogeny of the various subsets of cells which establish the system indicate a precisely coordinated sequence of reaction patterns to achieve an optimal situation at any given time during development. The developing mammalian fetus

must have available at and before birth a immune system functioning sufficiently to protect against invading microorganisms as well as against maternal lymphocytes sensitized against paternal alloantigens. At the same time there must be appropriate controls to prevent potentially damaging autoreactive processes. The picture that emerges from studies of the developing fetus and newborn strongly indicate the existence of reactivity patterns designed to deal with these requirements. There clearly does exist a gradual development of immune capacity in several cell lineages during gestation. This is followed by some quite dramatic changes in reactivity profiles subsequent to delivery. The maturation of the immune system after birth can be shown in many cases to closely parallel the normal physiological decay of strong elements of regulatory suppressor cells and inhibitory humoral factors. The fetus and newborn can thus be shown to contain humoral immunosuppressive substances of both endogenous and exogenous origin, as well as efficient suppressor-inducing cellular systems with the potential to interrupt invading aggressive T lymphocytes. It is likely that deeper understandings of how these naturally occurring suppressing factors and cells are induced into such effective functions may lead to positive applications in certain clinical situations where selective immune regulation is waranted.

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